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The Board of Editors of the Journal of the National Cancer Institute invites thoughtful speculative papers on cancer—its nature, origin, and development. This invitation is prompted by a desire to stimulate informed theorizing by those involved in cancer research or those who have considered the field from some other vantage point of science, since it is our conviction that speculative summaries have heuristic as well as historical and philosophical value.

It is hoped that the following article by Dr. E. E. Osgood may be the first of a series of hypotheses on neoplastic disease. Discussions of published hypotheses are also invited; they should be prepared according to the "Suggestions to Authors" on the inside back cover and submitted along with pertinent bibliography.

The chief mission of the Journal remains as before: the publication of original contributions to cancer research through laboratory and clinical investigations and statistical studies. The publication of any paper by the Journal does not imply its endorsement by the National Cancer Institute.

A Unifying Concept of the Etiology of the Leukemias, Lymphomas, and Cancers [1,2]

E. E. Osgood, *Division of Experimental Medicine, University of Oregon Medical School, Portland, Oregon*

At intervals it seems worthwhile to assemble as much information as possible in a particular field of investigation and to seek a logical inference from this evidence. The concept to be presented was formulated in this way in 1950. This concept seemed to unify and clarify the data then available on the nature of normal and malignant growth. Since the human mind tends toward bias, experiments, reading, and discussions with leaders in the field of growth and cancer were started to seek out any observation that might be incompatible with this hypothesis. Instead, additional evidence tending to support it was found. If correct, the hypothesis would indicate a redirection of much of the cancer research now in progress. Therefore, evidence both for and against it is presented, so that others may aid in testing its validity. This concept was mentioned only briefly in an earlier article (*1*).

Outline of the Concept

In the postnatal life of multicellular organisms, only the morphologically and chemically immature cells of a particular series divide. After one such cell divides, if each of the two daughter cells differentiated, no cell would be left for cell division. If both remained immature, to divide again, no differentiated cell would result (*2*). Therefore, one of the daughter cells must differentiate beyond the stage at which it is capable of division and ultimately die. The other must remain immature to divide again and be, for all intents and purposes, the same cell as the initial parent cell. This type of division leads to arithmetic changes in cell number. Only at long, unequal intervals (*3*) does the cell divide to form two cells that are both capable of division. These rare logarithmic divisions, of which only 4 or 5 occur, on the average, in all of postnatal life in man, constitute the basis of true growth (*4*). Therefore, each series of cells of any given type requires specific regulators to maintain in equilibrium the number of cells in the adult. A logical postulate which would provide an

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explanation is that with the majority of cell series the homeostatic regulator is an inhibitor of arithmetic cell division and is probably produced by the most chemically mature of the differentiating cells of that series. Somewhat earlier in the process of differentiation an inhibitor of the logarithmic division that constitutes the growth process must be produced. If this were the case, all that would be necessary for unlimited growth—in other words, malignancy—would be the absence of the inhibitor of logarithmic growth. The simplest mechanism which could explain this decreased production of inhibitor would be early death of the differentiating cell before it reached the stage at which inhibitor was produced. (*See* text-fig. 1.) In other words, malignancy is due not to the presence of something but rather to the absence of something, probably one or more enzyme systems, and it could be *any* enzyme system necessary for a normal life span of the differentiating cell.

Let us look at some of the implications of this hypothesis and see how the facts fit in with it.

Phylogenetic and Embryologic Evidence

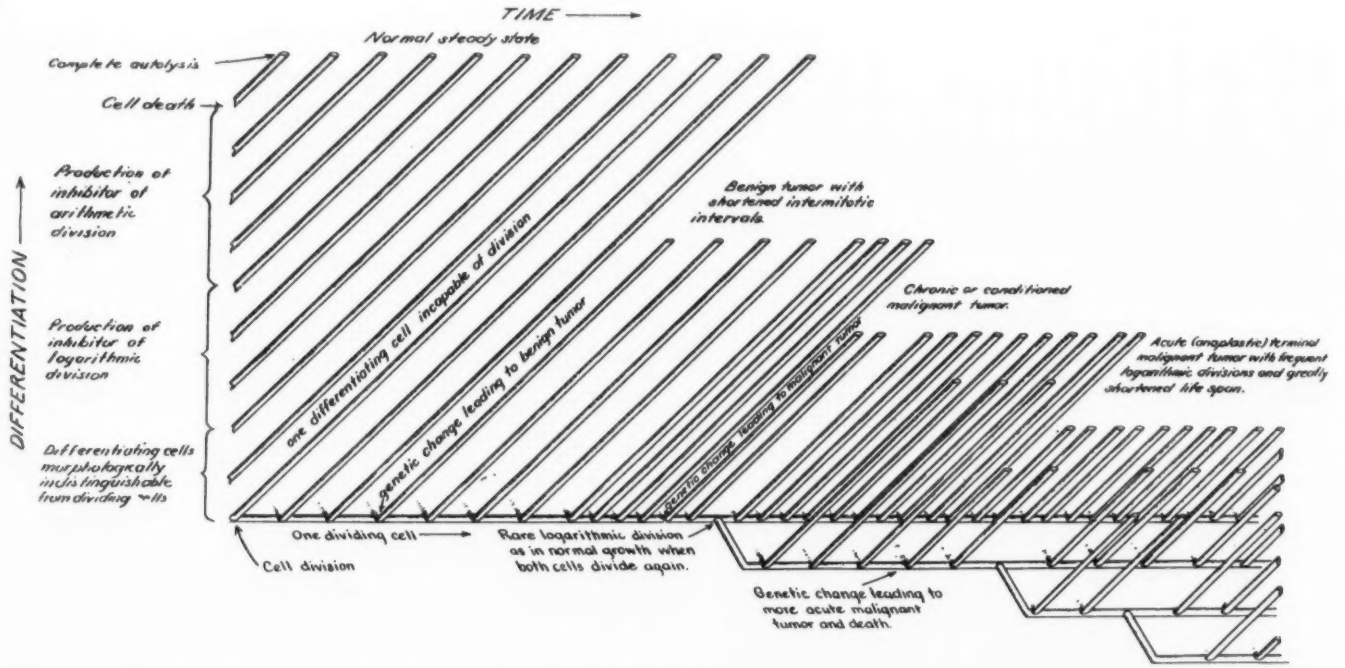
In the course of evolution, the unicellular organism preceded the metazoa. Almost all unicellular organisms, given a suitable environment, are capable of exponential growth, where doubling times are equal and growth is illustrated by a straight line on semilog paper. Such growth occurs in the first few days and for the first few cell divisions of the fertilized ovum (*5*), and is observed in the most acute leukemias and in the most rapidly growing tumors. During the rest of embryonic life and all postnatal life, the intervals between logarithmic divisions become progressively longer, and such growth can be described over long periods of time by straight lines on log-log,[3] not semilog, paper (*4*). According to the unifying concept, the capacity for unlimited exponential growth in the absence of its inhibitor of logarithmic growth is retained by each cell capable of division; the production of inhibitors became essential when multicellular organisms developed.

The production of short-range humoral agents that modify growth has analogues in the inducers and organizers of embryonic development.

Evidence That Differentiating Cells Produce Inhibitors

Removal of the mature cell from the environment should lead to a more rapid rate of cell division by decreasing the concentration of inhibitor, if this concept is correct. If one scrapes a bit of superficial epithelium from the surface of the skin or a mucous membrane, the rate of division of the underlying cells increases. Only the cells immediately subjacent are affected, indicating that the inhibitor must be relatively unstable and that it is not diffused throughout the body. If blood is withdrawn from a healthy individual, cell division increases in the marrow. Nothing

[3] Since the original publication (*4*), this has been confirmed in other species, such as Holstein cattle, rats, and rabbits.


TIME
Normal steady state
Complete autolysis
Cell death
DIFFERENTIATION
Production of inhibitor of arithmetic division
Production of inhibitor of logarithmic division
Differentiating cells morphologically indistinguishable from dividing cells
one differentiating cell incapable of division
genetic change leading to benign tumor
Benign tumor with shortened intermitotic intervals
Chronic or conditioned malignant tumor
genetic change leading to malignant tumor
Acute (anaplastic) terminal malignant tumor with frequent logarithmic divisions and greatly shortened life span.
One dividing cell
Cell division
Rare logarithmic division as in normal growth when both cells divide again.
Genetic change leading to more acute malignant tumor and death.

TEXT-FIGURE 1.—Graphic representation of unifying concept of the etiology of leukemias, lymphomas, and cancers. Visualize logarithmic division as a 3-dimensional graph with extension toward eye from plane of paper. Imagine graph extends indefinitely to *left*.

has been added. If one transfuses blood into a normal or anemic individual, the rate of red-cell division is decreased (*6*) until the erythrocytes have returned to the normal equilibrium number (*7*). In hemolytic anemias, where the cell life span is shortened, rapid multiplication of cells in the marrow occurs, even if there is a normal total-erythrocyte count, in the known absence of mature cells of the series. Crosby has shown that the marrow is capable of compensating when the life span is reduced to one sixth of normal (*8*). Leukemic and other malignant cells do not grow well *in vitro* in undiluted normal serum. However, all that is necessary to induce increased mitotic rates and even long-term growth is to dilute the serum with a simple balanced salt solution containing only nutrients characteristic of the general metabolic pool (*9*). This suggests the presence of some slightly more stable inhibitors that build up to and are maintained at a uniform concentration in the steady state and are specific for a particular cell series.

The regeneration that follows removal of parts of organs would be explained by a temporary decrease in the production of inhibitors; the characteristic transient overproduction of new cells (*10*) during regeneration of a part would be explained by the time lapse following restoration of normal cell numbers before the concentration of inhibitor reached equilibrium again. Removal of two thirds of the liver in one of a pair of parabiotic rats leads to increased cell multiplication not only in the liver of the operated animal but also in that of the parabiotic rat (*11*). Sham operation does not lead to such increased cell division. Again nothing has been added. This suggests that removal of a portion of the cell population prevents maintenance of the homeostatic level of a somewhat more stable inhibitor.

In the acute leukemias, the introduction of preformed inhibitor would account for the transient benefit observed with exchange transfusion (*12*).

This concept would explain the observations on conditioned and autonomous tumors (*13*), on the basis that the conditioned tumor had only a moderate shortening of the life span and could grow successfully only under the most ideal conditions, whereas a further shortening of the life span would lead to autonomy. It would also explain why tumors are more easily transplanted into the aqueous humor of the eye and the brain (*14*) than into other sites, since the first two are probably more protected from diffusion of the hypothetical inhibitors and are sites where the cells would tend to remain aggregated and tend to be less subject to attack by reticuloendothelial phagocytes.

There are, doubtless, many other inhibitors and stimulators of cell division in addition to the one produced in the most mature cell of the series.

The fact that some chronic leukemias, even when untreated, maintain a stable elevated leukocyte count over long periods of time could be explained by postulating a reduction in cell life span such that the normal concentration of inhibitor is maintained by a larger population of cells dying shortly after reaching the inhibitor-producing stage. In other

words, to produce the same amount of inhibitor as normal cells, a larger population is required when cell death occurs during the inhibitor-producing stage.

Evidence for Shorter Life Span of Cell in Malignancy

The differentiating cells, even in the most chronic malignant tumors, should have a shorter life span than the corresponding normal cells if this postulate is correct. This has been demonstrated to be the case in culture for cells of chronic granulocytic leukemia (*15,16*). It has not yet been demonstrated for most other types of malignant tumors, but the increased ratio of immature to more mature stages in such tumors strongly suggests that this is the case.

If this hypothesis is correct, the more the life span of the cell is shortened, the greater should be the deficiency of inhibitor, the more rapid the growth of the tumor, the greater the proportion of the less differentiated cells, and the earlier the death of the host. That this is the actual state of affairs is common knowledge and needs no documentation.

It would explain the prognostic value of Broders' (*17*) method of grading tumors, since the shorter the life span of the differentiating cells, the greater would be the proportion of the immature cells, the lower the inhibitor concentration, and the faster the growth of the tumor.

It would explain Harold Stewart's observation (*18*) that the earliest change detectable in experimental cancer of the gastrointestinal tract is evidence of cell death at the distal tip of the villus, where the most differentiated cells are located.

Evidence That Various Genetic Changes May Lead to Malignancy

According to the hypothesis, it is not a specific change that is necessary, but rather *any* change that leads to a shortened cell life span; this would account for the fact that different tumors originating from the same cell type, for example, different hepatomas (*19*), may be lacking in different enzyme systems.

The observations by Timonen (*20*), Hsu (*21*), and Levan and Hauschka (*22*) that aneuploid and even subhaploid cells occur in normal tissues[4] show that even such major deficiencies as must result from the loss of one or more chromosomes will not universally result in malignancy. In other words, it seems probable that only a very small proportion of the potentially malignant genetic changes eventually result in actual malignancy. A possible explanation is the need for an environment protected against diffusion of the inhibitor from the normal cells.

Every cell division offers a finite risk of some change in the genetic material that would cause the differentiating cell to lack an enzyme system essential for its normal life span. Malignant growths would occur, if this concept were correct, only when enough of these altered cells were congregated in an environment suitable for their growth, that

[4] We have observed such cells in normal marrow.

is, in a site protected from diffusion, so that division would not be prevented by inhibitor derived from the normal cells still present. Anything leading to an increased rate of cell division should increase this risk. The release or implantation of many altered cells should greatly increase the chance of metastasis (*23*) or successful transplant (*24*) of a tumor as compared to release or implantation of one or a few cells, by increasing the probability that a cell would reach an environment protected from diffusion from the circulating pool of inhibitor.

This concept would account for the age distribution of malignancies (*25*), for, according to this hypothesis, only a very small percentage of the changes in the genetic material giving rise to a potentially malignant cell would actually become manifest. Several cells would probably have to undergo this change in the same environment—relatively protected from diffusion from adjacent normal cells. In other words, some "carcinomas" *in situ* might never develop into true malignant tumors, since a second or third shortening of the life span might be necessary before uncontrolled growth could occur. The longer latent period before minor degrees of shortening of cell life span become manifest would explain the relatively low incidence of chronic leukemias in children, while very acute leukemias could occur at any age (*26*).

Other Observations Explained by This Concept

If the life span of a cell is somewhat shortened, it could be further shortened but not extended, which would account for the tendency of chronic malignant tumors, *e.g.*, chronic leukemia (*27*), to develop an acute terminal phase and of transplanted tumors to become more acute in their course (*28*). This would also explain the logarithmic-probability character of the survival curve in carcinomas (*29*), leukemias (*30*), and lymphomas, since death with most such tumors, according to this hypothesis, is not due to the initial tumor but to a tumor derived from it by a further shortening of the cell life span, which is a random event analogous to the decay of a radioactive isotope.

How does this concept account for the approximate doubling of survival time of leukemic patients given titrated, regularly spaced total-body irradiation (*27*) and for the increase in life span in the acute leukemias produced by continuous maintenance administration of inhibitors of cell division (*31*)? These agents act as substitutes for the absent inhibitor of cell division and, when given in such a way that a constant leukocyte count is maintained (*32, 33*), they reduce the total number of cell divisions occurring and thus reduce the risk of a further genetic change that leads to a still shorter life span of the differentiating cell. Unfortunately, all effective mitotic inhibitors so far discovered are also mutagens, and the chief advantage to be expected from determining the chemical structure of the normal inhibitor is that it is probably not mutagenic.

This concept would explain the apparent contradiction that both hyperplasia (*34*) and hypoplasia (*35*) predispose to the development of malignancy—hyperplasia by increasing the chance for occurrence, in adjacent

cells, of a sufficient number of genetic changes to lead to a shortened life span; hypoplasia by increasing the chance for such few genetic changes as may occur to become established, because of a low local concentration of inhibitor. Kaplan's (*36*) observation that nonirradiated thymus glands transplanted under the skin of totally irradiated mice give rise to an increased incidence of leukemias would be explained by the general reduction in inhibitor concentration.

The phenomena that have been called maturation arrest, dedifferentiation, and the hiatus leukemicus would be explained by the simpler hypothesis of early cell death, which accounts equally well for the characteristic morphologic appearance. This, to the author at least, seems more logical. Growth in the individual within the course of evolution has been a forward process and seldom, if ever, retraces its steps. The concept of dedifferentiation is somewhat like having an aged man retrace his growth process back to childhood. Assuming reproduction at an earlier age, an appearance analogous to that of dedifferentiation or maturation arrest would be present if everyone died in childhood. If maturation arrest really occurred, one would expect low metabolism of such resting cells. Instead, exactly the opposite is found—hypermetabolism and a very rapid turnover, which would certainly fit much better with rapid reproduction, development to a certain stage, and then death of the cell, as is postulated here. The hiatus leukemicus is analogous to skipping from the age of 10 to the age of 50 without any intermediate stage, which does not make sense. A more plausible explanation would be either a period of hypoplasia induced by therapy (*see* fig. 1 in reference *2*) or the presence of an acute monocytic leukemia with a myelophthisic blood picture simulating that of chronic granulocytic leukemia.

Objections to the Concept

The occasional successful transplantation of a malignant tumor with a single cell, already mentioned, is one objection. The fact that we have not isolated such a postulated inhibitor but have merely presented evidence for its existence is another. There are other workers who are far better prepared, with respect to both training and facilities, to attempt such isolation. One of the reasons for this paper is the hope that it may provoke thought and research along these lines. The major objection is that pernicious anemia and other hemolytic anemias with extreme shortening of the erythrocytic life span ought to be malignant tumors, and yet they have not been so regarded generally. Yet the bone marrow of a patient with untreated pernicious anemia demonstrates hyperchromatism, great variations in nuclear morphology and cell size, large nucleoli, large numbers of disintegrated cells, and bizarre mitoses and polyploidy—in other words, most of the morphologic characteristics usually identified with malignancy. The increased metabolism and rapid metabolic turnover seen in malignancies are also found. The one thing lacking is widespread metastasis to tissues outside the marrow, liver, and spleen. It was only when we found by the gradient-culture technique (*37*) that the erythrocytic series

required a very high gradient factor—in other words, a very low oxygen tension—that the explanation for this lack of metastasis became apparent. There are very few locations in the body suitable for metastasis of cells requiring low oxygen tension. It is tempting to speculate that this may have been an evolutionary protective mechanism and that malignant tumors of the erythrocytic series may have been much more common far back in the evolutionary series. Before the discovery of the missing factor, pernicious anemia had a 100-percent fatal outcome and ran a time course similar to malignant tumors. In pernicious anemia, replacement of B_{12} leads to the restoration of the normal life span of the cell and, as long as it is regularly provided, to an essentially normal life span of the individual. Perhaps this represents the first control of a malignancy and predicts the way other malignant tumors may fall to medical research, one at a time, by discovery of and replacement of the missing factor for a particular malignancy in a particular individual. We should think of an ecology of cells and microenvironments (*38*) within the body, as the ecologists do for species and external environments. Only some of these environments are suitable for the implantation of a particular type of malignant cell. Such microenvironments, as well as the number of cells reaching a site, probably have much to do with the distribution of primary and metastatic malignant tumors.

The rare recoveries in disseminated malignancy may represent conditions analogous to those in the hemolytic anemias, in which an extrinsic factor, such as lack of the prosthetic group of an enzyme, rather than a change in the genetic material of the cell, is responsible for the shortened life span of the differentiating cell.

This concept would account for the invasiveness of malignant growth on the basis of the competitive advantage of a differential rate of multiplication. That this is sufficient has been shown in tissue culture (*38*) and by many population experiments in ecology (*39*). The competition would probably be most effective against cells of the normal strain from which the malignant cells were derived because of need for the same nutrients, which would account for the fact that leukemic cells ordinarily displace all normal cells of the same series. Thus, in acute granulocytic leukemias all mature neutrophils disappear, and skin cancers often spread through the skin for a long time before they invade other tissues.

Lines of Cancer Research Suggested by This Concept

A major effort should be made to isolate, characterize chemically, and, if possible, synthesize the inhibitor or inhibitors, and this search should be made in the mature, normal cell rather than in the carcinoma cell. This study promises to be difficult and time-consuming and may, if successful, provide therapy for only one particular type of cancer at a time, but the isolation and characterization of such compounds as insulin and oxytocin suggest that it may, at least, be possible with the skills and facilities available. Further studies should be made, especially in tissue culture, of the interaction of the malignant cell and its normal

counterpart and of the effect of removing the mature cell of the series on the growth of the normal cells. Normal and malignant cells of the same type should be grown contiguous to each other, separated by micropore or other filters permeable to protein molecules, but not to the cells. If this theory is correct, the growth rate of the malignant cells should be temporarily slowed by the presence of contiguous normal cells, whereas, if other concepts that imply the presence rather than the absence of something in the malignant cell are correct, the normal cells might take on malignant characteristics.

If this unifying concept is correct, however, the only real control would come from finding a replacement for the enzyme system or systems missing in the malignant cell and from supplying the missing enzyme. This would probably control only one type of cancer of a particular cell type during continuous administration of the missing substance, because according to this theory there may be many different enzyme systems missing in different cancers of the same cell type. For example, while a common deficiency in chronic granulocytic leukemia is the absence of leukocyte alkaline phosphatase (*40*), we have found histologically and morphologically unmistakable granulocytic leukemia which gave a positive alkaline-phosphatase test. There are aleukemic granulocytic leukemias, chlorosarcoma-producing granulocytic leukemias, and eosinophilic, basophilic, and neutrophilic granulocytic leukemias—all probably having a different constitution of the genetic and enzymic material of the cell involved. Melanotic and amelanotic melanomas are other examples.

Unification of Current Concepts

The concept of somatic genetic change (*41,42*) is basic to this hypothesis, but it may be *any* genetic change leading to a shortened life span of the differentiating cells, not just some one specific change. Ectopic and embryonic-rest tissues and normal cells in tissue culture would show an increased incidence of malignancy because of the lack of adjacent normal cells or high dilution resulting in a low local concentration of inhibitor. The other factors, such as chemical carcinogens (*43*), heredity (*44*), ionizing radiation (*40*), virus (*45,46*), or virus-like agents (*47*), all serve as predisposing factors to the genetic change and thus become a unified part of the theory rather than competing concepts. Certainly, for particular tumors, each of the above is a well-established inducing factor, but for some malignancies several of these factors may be definitely excluded. Warburg's observation (*48*) would be explained if we assume that the cell capable of division retains the embryonic type of metablism. Then, the greater the shortening of the life span, *e.g.*, the more acute or anaplastic the tumor, the greater would be the proportion of the cells showing this type of metabolism. This hypothesis would explain the differentiation of somatic cells, which presumably contain all genes, and also the homeostatic control of cell multiplication on the basis of development by a process quite analogous to the process of evolution

of the species; namely, the survival of mutations inhibiting certain genes when such inhibition was favorable to survival of the organism.

Summary

A unifying concept of the etiology of the leukemias, lymphomas, and cancers is suggested which postulates that the fundamental alteration is *any* genetic change leading to lack of any enzyme system necessary for a full life span of the differentiating cell of a series. This chemically mature differentiating cell produces inhibitors of cell division. Lack of the inhibitors permits the emergence of the capacity for unlimited and uncontrolled growth that is present in the unicellular organisms and has been retained in the early dividing cell of every fundamental cell type in at least some stages of postnatal life. It does not displace current theories of the etiology of malignancy, but rather shows how all may be correct and have in common the fact that they predispose to the development of some change in the cell that leads to a shortened life span and hence to the lack of growth inhibitors.

This concept certainly does not simplify the cancer problem, but it does unify the many diverse facts now known.

It is presented not as an established fact but as a logical concept supported by much evidence and controverted by no information at present known to the author, with the hope that it may stimulate the thinking and research of others.

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The Cytochemical Localization of Esterase [1]

M. S. BURSTONE, *National Institute of Dental Research,*[2] *Bethesda, Maryland.*

This work deals with the synthesis and histochemical use of several new chromogenic substrates of the naphthol AS series for the demonstration of esterase. With frozen-dried tissues, these compounds enable precise histochemical localizations heretofore not possible with the simpler naphtholic substrates.

Methods

Synthesis of Substrates

Attempts to synthesize the substrates by heating the naphthols [3] with the appropriate acid chloride or anhydride in the presence of pyridine proved unsuccessful. Therefore the following procedure, based on a synthesis from the solid state, was evolved.

Naphthol AS-LC (the 2,5-dimethoxy-4-chloro-anilide) acetate (text-fig. 1) was prepared as follows: To a suspension of 5 gm. (approximately 0.012 *M*) of naphthol AS-LC in 50 ml. tetrahydrofuran was added an excess of acetic anhydride (4 ml.). A slight excess of 5 *N* NaOH (4 ml.) was then added with constant stirring until the solution became clear. The clear supernatant was poured into petroleum ether and the precipitate collected under suction. The precipitate was dried, dissolved in hot methanol, filtered, and recrystallized several times from cold methanol (m.w. 399.82).

Analysis.—calcd.: C, 63.08; H, 4.54; N, 3.50; Cl, 8.87
found [4]: C, 62.97; H, 4.76; N, 3.51; Cl, 8.92
m.p.: 138° C.

Naphthol AS-LC propionate and butyrate were also synthesized.

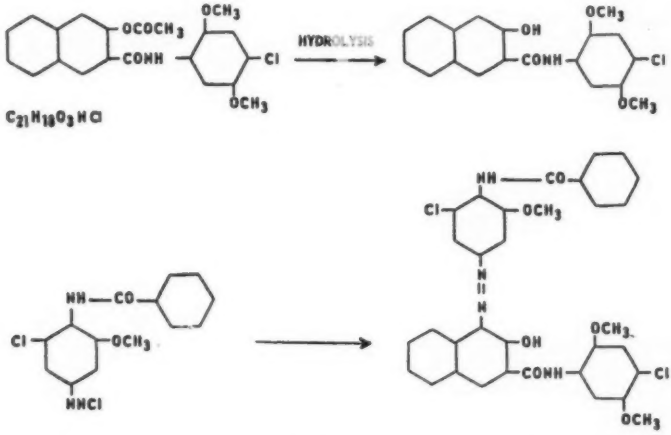
Naphthol AS-LC propionate (m.w. 413.8)
Analysis.—calcd.: C, 64.56; H, 5.18; N, 3.27; Cl, 8.3
found [4]: C, 64.32; H, 5.18; N, 3.35; Cl, 8.6
m.p.: 135° C.

[1] Received for publication September 4, 1956.
[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[3] The naphthols were furnished through the courtesy of the Pfister Chemical Works, Ridgefield, N. J.
[4] Microanalyses done by Dr. W. Alford and group.

Naphthol AS-LC butyrate (m.w. 427.8)
Analysis.—calcd.: C, 63.84; H, 4.87; N, 3.39; Cl, 8.57
found [5]: C, 63.65; H, 4.99; N, 3.29; Cl, 8.64
m.p.: 133° C.

Naphthol AS-E acetate (the 4-chloro-anilide) was prepared by a similar procedure.
Analysis.—calcd.: C, 67.16; H, 4.15; N, 4.12; Cl, 10.44
found [5]: C, 66.94; H, 3.91; N, 4.24; Cl, 10.46
m.p.: 178° C.

In addition several similar compounds were synthesized. These included the isomer of AS-E, AS-MCA (2-chloro-anilide), and also several chloro-toluidides (AS-TR and AS-KB). All of this group, except the AS-E acetate, were poorly hydrolyzed and were considered to be unsatisfactory for histochemical use.



TEXT-FIGURE 1.—Hydrolysis of naphthol AS-LC acetate and coupling with Corinth LB salt.

Histochemical Procedure

Both acetone-fixed and frozen-dried double-embedded (celloidin and paraffin) rat tissues were used. They included liver, kidney, pancreas, spleen, intestine, stomach, testis, and tongue. In addition, several human neoplasms were studied.

With the LC acetate the following procedure was employed on the double-embedded material: Twenty to 30 mg. of substrate were dissolved in 2 to 4 ml. dimethylformamide (DMF), 5 to 10 ml. ethylene glycol monoethyl ether (ethyl cellosolve) were added, plus 10 ml. 0.2 *M* buffer (tris, *p*H 7.1—at this *p*H coupling with the garnet salt produces a beautiful violet color), and sufficient distilled water to make 50 ml. of

[5] Microanalyses done by Dr. W. Alford and group.

solution. Twenty to 30 mg. diazonium salt [Garnet GBC,[6] diazotized 4-*o*-tolylazo-*o*-toluidine (*o*-aminoazotoluene); or Corinth LB,[7] diazotized 2-methoxy-1-benzoylamino-6-chloro-4-aminobenzene] were added and the mixture was shaken, filtered, and used at room temperature (24° C.). (Since filtration may be rather slow, the substrate solution was usually first poured into the coplin jar, and the diazonium salt, in a few ml. buffer, was then filtered into the jar.) Incubation times usually range from 10 to 40 minutes. With rat pancreas (double-embedded) the incubation time is 10 to 15 minutes. With acetone-fixed tissues a higher solvent ratio, using 5 ml. DMF and 15 ml. cellosolve, has been used successfully and in some tissues gives good localizations in a matter of minutes. Acetone-fixed tissues were incubated for periods up to 1 hour. The wider latitude of activity of the frozen-dried tissues may necessitate variation in the substrate-solvent ratio. This is particularly the case with single-embedded frozen-dried tissue, since too high a solvent ratio will cause diffusion of the final dye product.

The LC propionate and butyrate were used only with frozen-dried double-embedded pancreas (2 ml. DMF and 10 ml. cellosolve). The AS-E acetate was dissolved in 2 ml. methyl ethyl ketone and 5 to 10 ml. Carbowax 200. Twenty to 30 mg. Violet B salt (diazotized amino-methyl-*m*-benzanisidine) were added, and for longer incubations 1 or 2 drops of a 20 percent solution of Tween 20 were used. The Tween serves as a dispersing agent. All tissues were incubated at *p*H 6.8.

Stained sections may be counterstained with hematoxylin and mounted in glycerol gelatin. They may also be mounted in an 80 percent alcoholic solution of polyvinyl acetate (*1*).

Observations [8]

With the LC-acetate substrate the distribution of activity was essentially the same as observed with other naphtholic substrates (*2*). However, exceedingly sharp localizations were obtained with relatively short incubation periods. Even under the highest magnification there was no evidence of diffusion. The pattern of staining was usually granular and appeared to be associated with mitochondria.

In the kidney, certain spherical bodies (fig. 1) stained intensely. These structures appeared to be similar to those described by Sjöstrand (*3*) and also by Holt (*4*) and Pearse (*5*) with the indoxyl acetate method. The basal portion of the kidney tubules showed the characteristic striated pattern that was formed by the arrangement of the granules. Many of these granules were barely resolvable with the highest optical magnification.

The pancreas revealed a very precise pattern of activity localized to zymogen granules (fig. 2).

[6] General Dyestuff Corporation, N. Y.

[7] Verona Chemical Company, Union, N. J.

[8] Preliminary observations with the LC-acetate method on cold formalin-fixed frozen sections (rat kidney and liver) revealed good localizations. The incubation time was 25 minutes and the substrate was dissolved in 2 ml. DMF and 5 ml. cellosolve plus the usual quantity of buffer.

Serous glands of the tongue revealed a precise zone of activity with some unstained intra-acinar vacuoles (fig. 3). Mast cells also stained intensely.

Even in some acetone-fixed tissues, precise localizations were obtained (fig. 4).

The human tumors studied included an oral epidermoid carcinoma and a poorly differentiated lung tumor. In both of these lesions esterase-positive cells showed a granular distribution of the enzyme. With simpler substrates, such as naphthyl acetate, the distribution is rather homogeneous, suggesting the possibility of diffusion. Further cytologic study of tumors with naphthol AS-LC acetate is indicated in order to evaluate the nature and significance of the reactive areas.

The LC propionate and butyrate gave the same localization in the pancreas as seen with the acetate, and with the same incubation time. Other tissues should be evaluated with these substrates for comparative studies.

The naphthol AS-E acetate does not have any advantage over the AS acetate of Gomori (*2*). The AS-E compound is more slowly hydrolyzed, and the localizations appear no better than with naphthol AS acetate. The procedure is useful in that the diazonium salt used has a low decomposition rate.

Discussion and Summary

The synthesis and histochemical use of several new esterase substrates of the naphthol AS-LC series which are capable of giving very precise localizations are described.

Because of the high substantivity of the naphthols used, a larger amount of substrate and solvent may be used in order to approach more closely the optimal reaction velocity of the Michaelis-Menten equation. With less substantive naphthols a large amount of solvent will simply remove the final dye product from the section. The relative solubility of the substrate as contrasted with the marked insolubility of the released naphthol would account for the successful application of the LC compound for cytochemical localizations.

The use of this and similar substrates for precise localizations may find application in bacteriology and electron microscopy, as well as cytochemistry.

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PLATE

PLATE 15

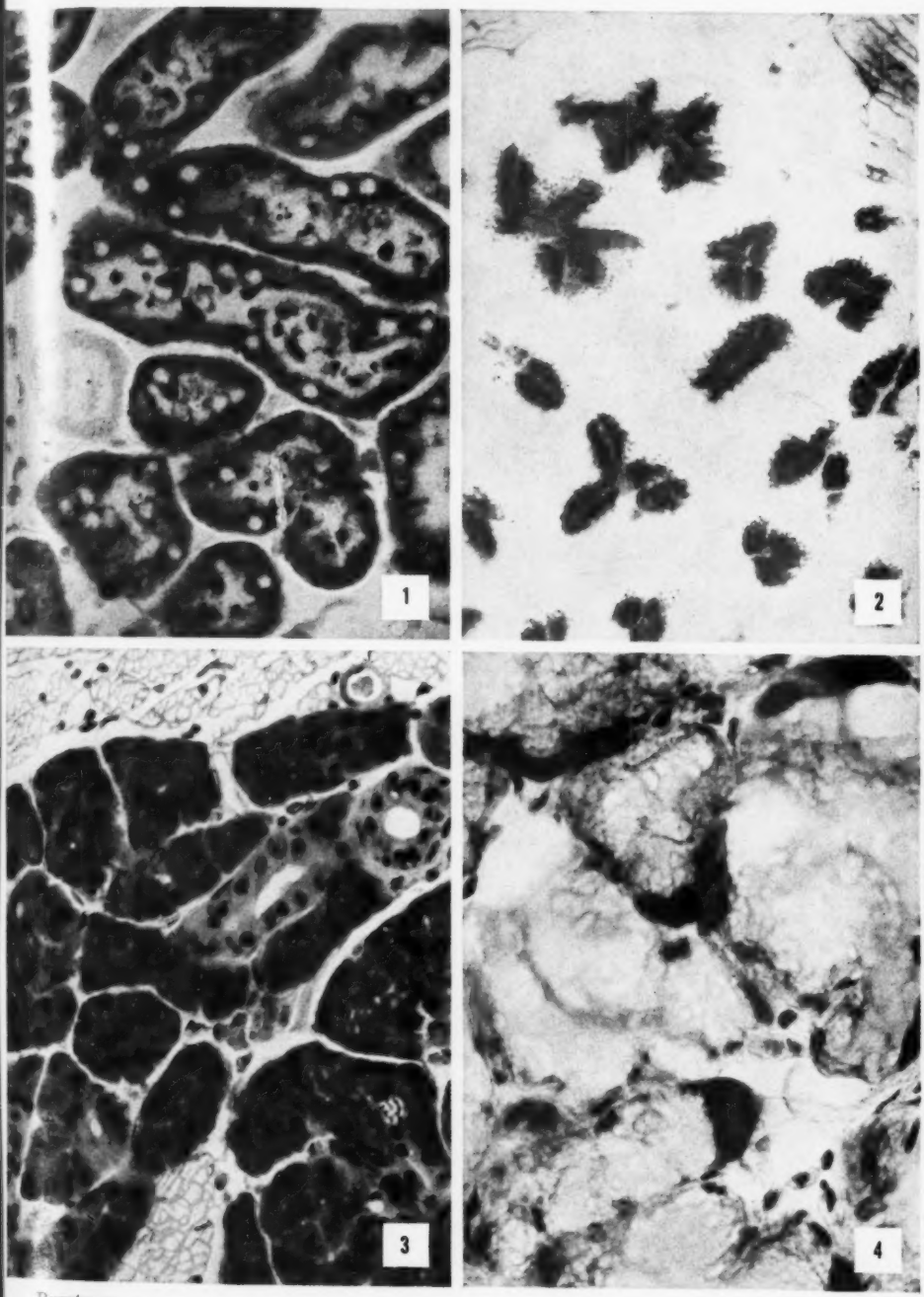
FIGURE 1.—Frozen-dried rat kidney, AS-LC method. There is intense staining of larger spherical bodies and also in the basal portion of the tubules. × 700

FIGURE 2.—Frozen-dried rat pancreas, AS-LC method. There is discrete staining of zymogen granules. × 1040

FIGURE 3.—Frozen-dried rat tongue, serous lingual glands. Hematoxylin counterstain. × 700

FIGURE 4.—Acetone-fixed rat tongue. AS-LC method, with hematoxylin counterstain. Activity is present in demilune cells of mucous gland. × 700

Photomicrographs taken by John T. McGuire.



Burstone

Histopathologic Studies of Peritoneal Implantation and Lung Metastasis at Different Stages of the Gradual Transformation of the MC1M Mouse Sarcoma into Ascites Form [1,2]

NILS RINGERTZ, EVA KLEIN, *and* GEORGE KLEIN,[3]
Institute of Pathology and Institute for Cell Research and Genetics, Karolinska Institutet, Stockholm, Sweden

There is considerable variation in the growth pattern of various mouse tumors in the peritoneal cavity. Hitherto, one aspect of this variation has been studied particularly; namely, the characteristic referred to as convertibility to the ascites form (A-form). According to its definition (*1*) this means the capacity of intraperitoneally inoculated tumor cells to maintain themselves and to increase in number in the peritoneal fluid, reaching there a high absolute number and relative concentration (of the order of 10^8 to 10^9 tumor cells per mouse, the relative proportion of tumor cells being usually between 60 and 95%). When tested by intraperitoneal inoculation of comparable amounts of minced tumor tissue, different tumors may behave differently, sometimes even in spite of similar morphologic and genetic origin (*2*). There are some tumors that grow in the ascites form immediately and without difficulty ("immediate conversion"), while others give rise only to solid tumors and infiltrations, accompanied by peritoneal exudates containing a comparatively small number of tumor cells, mostly desquamated (usually of the order of 10^4 to 10^6 per mouse, their relative proportion being between 1 and 10%—in many cases even below 1%). Some tumors of this second category can still be converted to A-form by prolonged serial transfer of peritoneal exudate ("gradual conversion"), while others are refractory to such attempts and remain unchanged through long periods of time.

The presence and the number of suspended tumor cells in the peritoneal fluid, interesting in themselves, represent only one aspect of a complex situation. The question arises of the extent to which tumorous infiltration of intra-abdominal tissues complicates the picture. In particular, it would be important to know whether there is any direct or inverse relationship in the capacity of different tumors to grow in peritoneal fluid and in solid tissues, and whether they exhibit any morphologic difference in the process of infiltrating solid tissues. As a first experimental approach to this problem, we felt that it would be unwise to compare unrelated tumor

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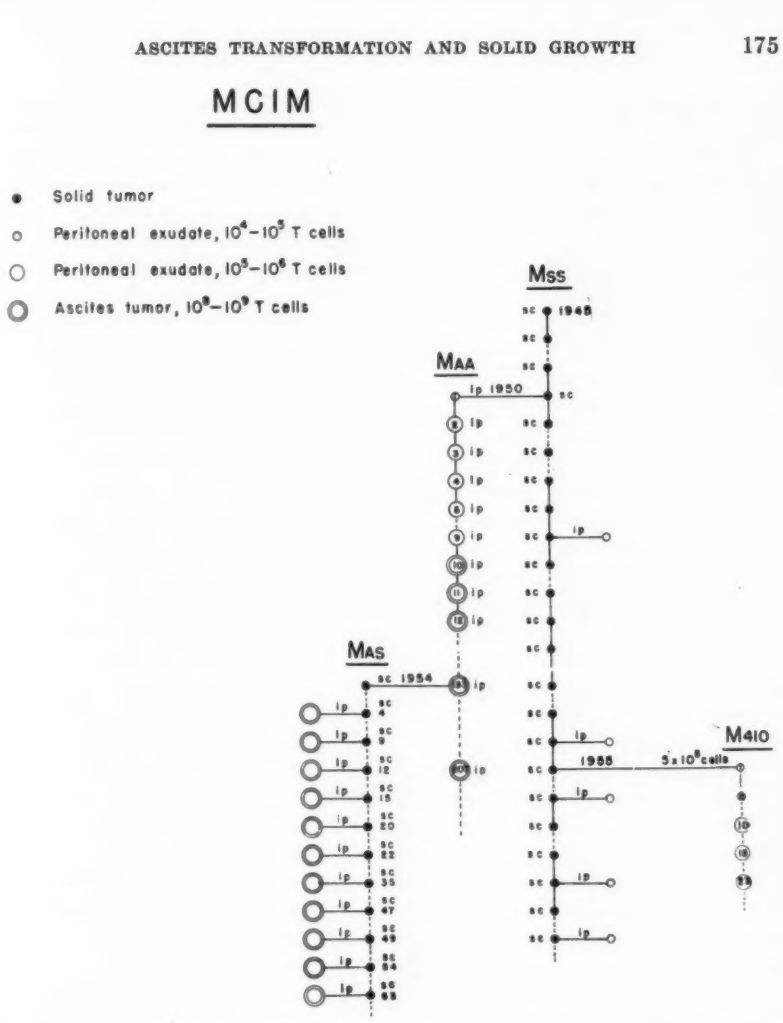
[2] This work was supported by grants from the Swedish Cancer Society.
[3] For valuable technical assistance, our thanks are due to Miss Marianne Jedeur-Palmgren and Mr. Karol Swiech.

strains varying in convertibility to A-form. Different neoplasms may differ in a great many characteristics and, as for intra-abdominal infiltration, even established ascites tumors may show great differences. Some of them are characterized by early and extensive infiltration, while others grow primarily in the peritoneal fluid and infiltrate only later. All intermediate stages exist. To study the correlation between infiltrative growth and proliferation of suspended tumor cells we have endeavored to avoid such intertumoral variation and preferred to compare different sublines of the *same tumor*. This was made possible by the availability of solid tumors, *gradually* convertible to A-form, such as the MC1M sarcoma (*1*). Representative sublines were selected before, during, and after conversion to A-form and also after prolonged subcutaneous passage of the converted ascites line. The relation between the number of tumor cells in the peritoneal fluid and the tumorous infiltration of various intra-abdominal tissues was then studied with the different sublines after the intraperitoneal inoculation of a standard cell number. In addition, the lungs of mice bearing intramuscular tumors were studied histopathologically and tested by inoculation, in order to compare the metastasizing ability of the various sublines. Finally, their growth in the subcutaneous space was compared histologically.

Materials and Methods

Mice.—For all experiments, C3H/StKl mice were employed or their F_1 hybrids, produced by outcrossing C3H/StKl with 1 of the following 3 inbred strains: A/StKl, DBA/HaKl, and C57BL/HaKl (*3*). An inbred nucleus of all 4 strains is maintained by strict single-line brother × sister mating, and animals used for experiments are never permitted to deviate from the nucleus by more than 2 brother-sister generations. The mice are kept on a standard checker diet which, with drinking water, is available *ad libitum*. Animals of both sexes were used at the age of 3 to 5 months with a body weight of 20 to 30 gm.

Tumor lines.—The MC1M sarcoma, induced by methylcholanthrene in a C3H mouse by Miller and associates (*4*), was used for all experiments. The transplantation history of the various sublines used appears in text-figure 1. Line M_{ss} is the original solid line, kept by serial subcutaneous transfers. Intraperitoneal inoculation of this neoplasm does not result in the multiplication of free tumor cells in the peritoneal fluid, *i.e.*, an ascites tumor, but rather in a peritoneal carcinomatosis, followed by the formation of a secondary exudate that contains only a comparatively small number of desquamated tumor cells (of the order of 10^4 to 10^6). By 10 generations of serial intraperitoneal-exudate transfer the tumor was converted into A-form and the ascites line M_{AA} was developed (*1*). The growth characteristics of this line have been described elsewhere (*5,6*); for the present purposes it is sufficient to mention that inoculation of 10^5 cells or more to C3H mice results, in all animals, in typical ascites tumors containing $3–5 \times 10^8$ free tumor cells at the peak of development. With this line, tumor cells start multiplying directly in the peritoneal fluid after inoculation and their increase does not depend on the presence of solid-tumor



TEXT-FIGURE 1.—Transplantation history of the MC1M sarcoma sublines used for the present experiments. For M_{AA}, M_{AS}, and M_{410}, the figures denote the number of serial passages.

implants. Subcutaneous inoculation of ascitic fluid from line M_{AA} results in solid tumors, designated M_{AS_1}. From such solid tumors a new transfer line was established by serial subcutaneous transplantation, called M_{AS_n}, where n denotes the number of serial transfers since the line diverged from the ascites form. In contrast to M_{SS}, this line possesses the capacity to grow immediately in the peritoneal fluid. All 3 lines grow progressively in 100 percent of C3H mice, irrespective of sex. The same applies to F_1 hybrids with C3H as 1 parent. They do not grow regularly in unrelated genotypes. In F_2 hybrids, produced by outcrossing C3H to A, they all

grow in approximately 75 percent, indicating the requirement of 1 histocompatibility factor with respect to this particular hybrid combination.

In addition to the 3 main lines, a fourth subline designated M_{410}, was used for some of the experiments. This subline was derived from M_{88} by the intraperitoneal inoculation of 5×10^5 tumor cells, a dose 40 to 80 times lower than usual. In conformity with previous findings (*2,6*), this decrease of the initial inoculum size reduced the convertibility of the tumor to a considerable extent. In spite of 23 intraperitoneal-exudate transfers the line is still not entirely converted to A-form, although the concentration of free tumor cells has increased lately. Data on its growth will be given in the section on results.

After the intraperitoneal inoculation of 10^6 tumor cells from the various sublines the median survival time of C3H mice varied between 12 and 15 days. There was no significant difference between the 4 sublines.

Methods.—Artificial suspensions were made from solid tumors of the various sublines by forcing the freshly removed, ice-cooled tissues through a series of stainless-steel screens, as described by Kaltenbach (*7*). Care was taken to remove all macroscopically necrotic tissue. The material was suspended in Krebs-Ringer-phosphate (*p*H 7.4), containing 0.25 percent gelatin and 0.5 percent glucose. The cells were counted in a hemocytometer after dilution with a 0.05 percent eosin solution, as described by Schrek (*8*). One million eosin-unstained cells were inoculated intraperitoneally, intramuscularly, or subcutaneously. Subsequently, the mice were sacrificed at regular intervals. Intraperitoneally inoculated animals were used for determination of the total number of free tumor cells by the dye-dilution method of Révész and Klein (*9*), and a large number of tissue samples from the abdominal cavities were examined histologically. Representative portions of the abdominal wall, omentum, mesentery, retroperitoneal fat, pancreas region, the connective and fatty tissue surrounding the ovaries or testes, etc., were fixed in 10 percent formalin, embedded in paraffin, and sectioned at 4 to 5 μ. The sections were stained by hematoxylin and eosin and hematoxylin and van Gieson.

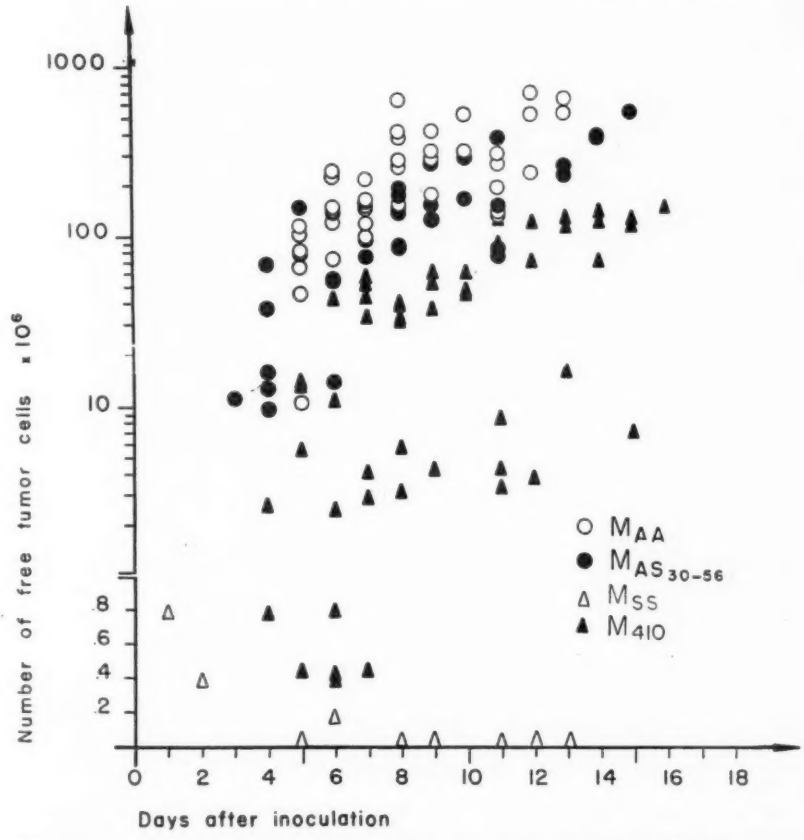
Lungs were fixed and embedded in the same way and longitudinal sections were cut from each lobe, at least 6 from each lung. Subcutaneous implants were localized, beginning 12 hours after inoculation, and studied in sections cut perpendicular to the epidermal surface.

Results

Number of Free Tumor Cells in Peritoneal Fluid

The total number of free tumor cells in the peritoneal fluid was determined as a function of time after the inoculation of 1 million eosin-unstained cells of the 4 sublines indicated in text-figure 1. Representative growth curves are shown in text-figure 2. As in previous findings, M_{88} was characterized by the absence of free-cell growth in suspension form and the number of tumor cells in the peritoneal fluid decreased soon after inoculation. All animals died with extensive solid-tumorous infiltration

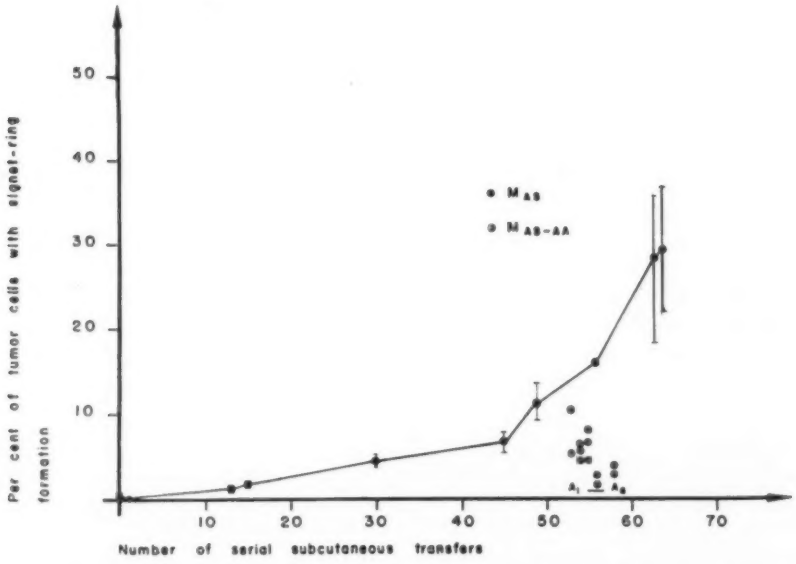
of the peritoneal cavity, as will be described below, accompanied by hemorrhagic exudates poor in tumor cells (fig. 1). In contrast, M_{AA} cells multiplied rapidly in the peritoneal fluid, behaving as in a typical ascites tumor (fig. 2). The growth of M_{AS} was of particular interest, since its behavior has a bearing upon the question of whether the cellular change leading to ascites transformation is of a permanent nature or whether it can be reversed by propagation in the original solid environment. Previously, M_{AS} was tested after 15 and 20 subcutaneous generations, and it behaved similarly to M_{AA}. Since then it has passed through a series of additional solid transfers. Its growth was tested after 45, 49, and 56 solid generations and, as shown in text-figure 2, it still maintains the capacity to grow in A-form, in contrast to the original M_{SS} line. This proves the permanence of the change. However, in comparison



TEXT-FIGURE 2.—Total number of free tumor cells in the peritoneal fluid as a function of time after the inoculation of 1 million eosin-unstained cells, derived from the various sublines of the MC1M sarcoma. Each point represents 1 mouse. Arithmetic scale 0 to 10^6; logarithmic scale 10^6 to 10^9.

with M_{AA}, M_{AS} has undergone still further modification, not apparent from the growth curve. Since the 30th solid transfer generation, an increasing proportion of free tumor cells undergoes excessive vacuolization and, in the extreme, appear similar to so-called signet-ring cells. It is quite clear that these vacuolated cells are tumor cells and not phagocytic cells of the host. All stages of transition can be seen from tumor cells with small vacuoles to the extreme condition. Even in the latter the nuclear structure is typical of the MC1M sarcoma. The chemical composition of the vacuolar contents is unknown as yet; tests for mucoid substance have been negative. This excessive vacuolization happens only when the line is tested by intraperitoneal inoculation, and no change has occurred in the cellular morphology of the solid subcutaneous tumor. Text-figure 3 shows the mean frequency of the extreme signet-ring type, expressed as the average of 10 to 14 samples collected between days 5 and 12 after the inoculation of 1 million tumor cells from different subcutaneous transfer generations of M_{AS}. No consideration has been given to less excessively vacuolized cells in text-figure 3. They usually parallel the frequency of the signet-ring cells, although they are more numerous. Figure 3 illustrates the change.

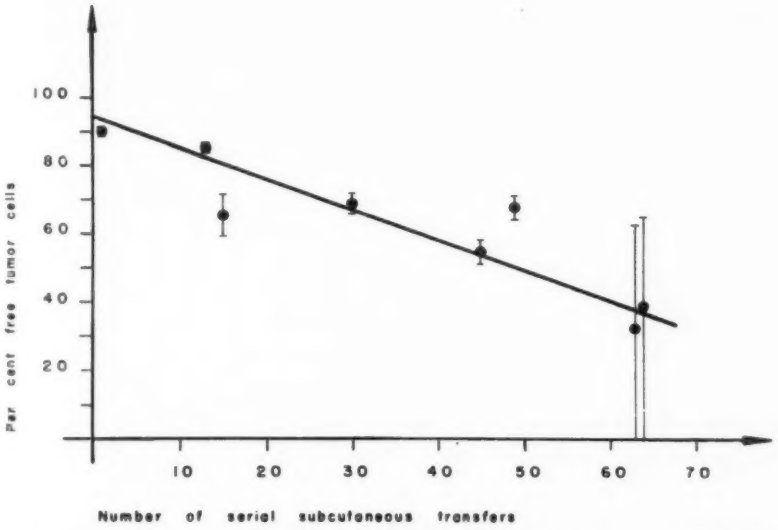
The signet-ring cell could be brought to disappearance by a few generations of uninterrupted intraperitoneal transfer of ascitic fluid, as shown



TEXT-FIGURE 3.—Mean frequency of tumor cells showing excessive signet-ring formation, expressed as percentage of all tumor cells in the peritoneal fluid. Each point represents an average based on 10 to 14 mice, sampled between 5 and 12 days after the inoculation of 1 million tumor cells from different serial subcutaneous transfer generations of M_{AS} (*cf.* text-fig. 1). Cells derived from the 52nd generation were also carried intraperitoneally through 6 transfers. The results are shown as M_{AS-AA}.

in text-figure 3. The product of this procedure was quite indistinguishable from the original M_{AA} subline.

Simultaneously with the development of signet-ring cells, the average frequency of free tumor cells in the peritoneal fluid showed a highly significant progressive decrease (text-fig. 4). As shown in text-figure 2, the total number of free tumor cells did not change to any detectable degree. It follows that their decreased relative proportion was due to an absolute increase of the total number of nontumorous cells in the fluid. The mean number of nontumorous cells is of the order of 20 million in the M_{AA} ascites line between the 5th and 12th day after the inoculation of 1 million tumor cells; the corresponding figure of $M_{AS_{49}}$ lies between 56 and 69 million. There was a statistically probable correlation between the frequencies of signet-ring tumor cells and of nontumorous cells in the fluid ($n=68$, $r=0.305$, $0.02>P>0.01$). When ascitic fluids derived from $M_{AS_{52}}$ were transferred serially in the peritoneal cavity through 6 passages, the increased inflammatory response subsided concomitantly with the disappearance of the signet-ring cells.



TEXT-FIGURE 4.—Mean frequency of all free tumor cells in the peritoneal fluid during the 5- to 12-day period after the inoculation of 1 million tumor cells from different subcutaneous transfer generations of M_{AS}. Each point is based on 10 to 14 mice. The range of the values is indicated.

The fourth subline, M_{410}, was also studied with regard to growth in the peritoneal fluid. As indicated by the data in text-figure 2, there was an increase in the number of free tumor cells after inoculation, but much smaller than with the established ascites line. There was also a very high variability between individual mice.

Histopathology of Peritoneal Implantation

The findings are summarized in table 1. This table is based on 7 series of experiments: 1 with M_{SS}, 2 with M_{410}, 2 with M_{AA}, 1 with $M_{AS_{30}}$, and 1 with $M_{AS_{56}}$. Sixteen to 22 mice were inoculated in each series and killed in pairs at different times after inoculation. Since the findings on parallel series were closely similar, they were pooled. Each figure in table 1 is thus based on 2 to 4 mice, studied individually but evaluated and tabulated collectively.

TABLE 1.—*Histologic findings regarding intra-abdominal tumorous infiltration and local inflammatory reaction after intraperitoneal inoculation of 1 million tumor cells from various MC1M sublines*

Days after inoculation	M_{SS}		M_{410}		M_{AA}		$M_{AS_{30-56}}$	
	Tumorous infiltration*	Inflammatory reaction†	Tumorous infiltration*	Inflammatory reaction†	Tumorous infiltration*	Inflammatory reaction†	Tumorous infiltration*	Inflammatory reaction†
1	−	−						
2	+	−						
3					−	−	+	+
4	++	−	−	−	±	+	+	+
5	++	−	+	±	+	+	+	+
6	++	±	+++	−	+	+	+	++
7	+++	−	+++	−	+	++	+	++
8	+++	−	+++	±	+	++	+	++
9	++++	−	++++	−	+	++	+	++
10	++++	−	++++	−	+	++	+	++
11	++++	−	++++	−	+	++	+	++
12	++++	−			+	++	+	++
13	++++	−			+	++		

* *Tumorous infiltration:*
± Groups of inflammatory cells, presence of tumor cells uncertain.
\+ Small foci of tumorous infiltration, with largest diameter less than 0.25 mm.
++ Implants of tumor tissue between 0.25 and 1 mm. in diameter, or very numerous smaller implants.
+++ Implants between 1 and 5 mm. in diameter.
++++ Implants with a diameter of 5 mm. or more.

† *Inflammatory reaction:*
\+ Small subperitoneal foci of inflammatory cells, mostly lymphocytes.
++ Confluent lymphocytic infiltration, extending through larger areas.

For each mouse the extent of growth in the solid tissues adjacent to the peritoneal cavity was evaluated from histologic sections, comprising numerous pieces of omentum, mesentery, retroperitoneal tissue, and parietal peritoneum. The grading principle employed is described in the footnotes to table 1. Particular attention was given to morphologic signs of good or poor vitality of the tumor cells and to the presence or absence of inflammatory infiltrations in or around the implants.

As shown in table 1, a pronounced difference was found between sublines M_{SS} and M_{410}, on the one hand, and sublines M_{AA} and $M_{AS_{30-56}}$ on the other. The former group displayed extensive solid-tumor growth, beginning 4 to 6 days after inoculation and reaching very considerable dimensions by the 9th day. The two sublines of the latter group started to produce recognizable implants at a similarly early stage but they did not continue

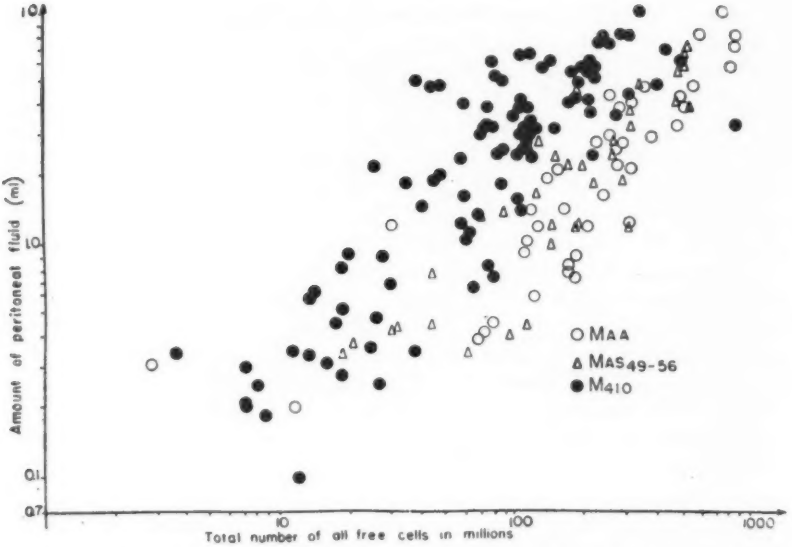
to grow on a like scale and failed to produce large solid-tumor masses subsequently. The gross difference is illustrated by figures 4 and 5.

There was a marked difference between the 2 subline groups also with regard to finer histologic details. Early implants of M_{AA} and $M_{AS_{30-56}}$ can be characterized as very superficially located small nodules, containing rather faintly stained tumor cells with slightly blurred contours and with few or no mitotic figures. A conspicuous infiltration of inflammatory cells of the lymphocytic and monocytic type was regularly detectable at the base of the nodule (fig. 6). The most extensive implants found at later stages in these series presented the picture of small omental plicae with a continuous, superficial covering of rather faintly stained, sometimes vacuolized tumor cells, the center of the tissue being densely infiltrated with lymphocytes and monocytes (figs. 7 and 8). At the latest stage of the growth cycle only small implants were found, containing few visible tumor cells in the superficial layer, surrounded by a very pronounced inflammatory infiltration (fig. 9). Signet-ring cells were quite common in implants of $M_{AS_{30-56}}$, while M_{AA} did not show any similar tendency.

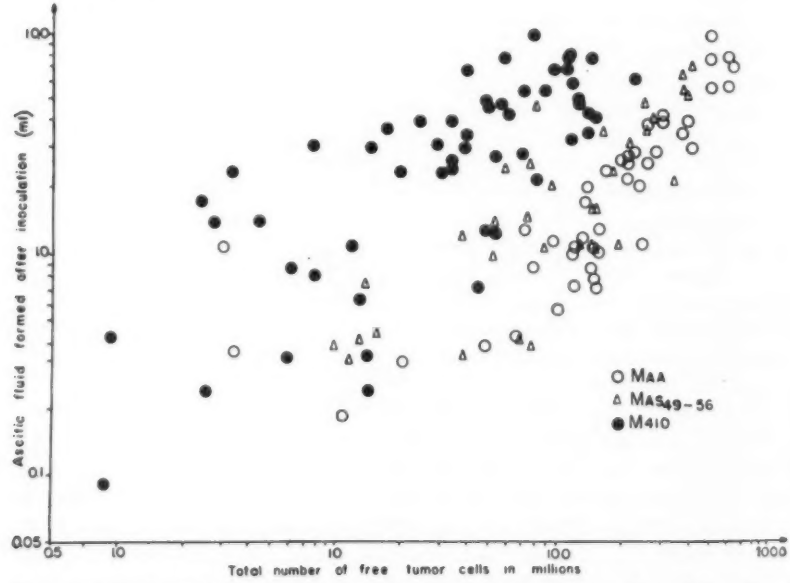
Implants derived from sublines M_{88} and M_{410} presented an entirely different appearance already at the earliest observed stage. The tumor cells were seen to infiltrate deeply into the subperitoneal tissue, even in very small nodules, their nuclei were stained well and had visible nucleoli, cell outlines were sharp, and mitotic figures were frequent (fig. 10). Inflammatory infiltration was entirely absent with the exception of 1 mouse in the M_{88} series and 2 in M_{410}. At a more advanced stage there was a very extensive tumorous infiltration, consisting of a densely cellular neoplastic tissue with mostly well-stainable, sharply outlined cells and many mitotic figures (fig. 11). In the central parts of the largest nodules there were often more or less extensive patches of necrotic tissue. Such areas were sharply limited against vital tumor tissue by a reaction zone of polymorphonuclear leukocytes. No inflammatory reaction of the lympho-monocytic type was ever seen in these border zones of necrotic and normal tissue. Since the inflammatory reaction was thus of a different nature than in the other 2 sublines and presented the picture that usually accompanies tumor necrosis due to circulatory disturbances, it was not recorded in table 1.

Amount of Peritoneal Fluid

Simultaneously with the determination of the total number of free tumor cells in the peritoneal fluid and the histologic studies, the total amount of the peritoneal fluid in sublines M_{AA}, M_{AS}, and M_{410} was also estimated by the dye-dilution method described by Révész and Klein (*9*). Text-figure 5 shows the results plotted against the total number of all free cells, while the relationship between accumulation of fluid and the number of free tumor cells is shown in text-figure 6. Similar determinations were not carried out with M_{88} because of the considerable inaccuracy involved in determination of the very low cell numbers.



TEXT-FIGURE 5.—Relationship between the total number of free cells (tumorous+non-tumorous) in the entire peritoneal fluid and the volume of ascites in sublines M_{AA}, M_{AS}, and M_{410}.



TEXT-FIGURE 6.—Relationship between the number of free tumor cells and the volume of ascitic fluid formed after the intraperitoneal inoculation of cells from sublines M_{AA}, M_{AS}, and M_{410}. The ordinates represent the total amount of ascites after subtraction of the average volume of peritoneal fluid in normal mice (0.12 ml., ref. *5*).

The results indicate that the amount of fluid per free cell and also per free tumor cell is regularly higher with M_{410} than with M_{AA} and M_{AS}. With M_{SS}, it would be even higher than with M_{410}, due to consistently low cell numbers accompanied by ascites of a magnitude grossly similar to the other sublines. With M_{SS} and M_{410}, the fluids were also more regularly and more intensely hemorrhagic than with M_{AA} and M_{AS}.

Dissemination of Tumor Cells to the Lungs from Intramuscular Tumors

Previously, it was found by one of us (*6*) that intramuscularly inoculated M_{AS} cells appear in the lungs earlier and more regularly than cells of the M_{SS} type, when compared by biologic testing. Presently these experiments were repeated and supplemented with histologic examination.

One million eosin-unstained M_{SS} or M_{AS_1} cells were inoculated intramuscularly into the right thigh. The animals were killed at successive intervals and their lungs were used for histologic examination or for bio-

TABLE 2.—*Tumor transmission with lung tissue from mice bearing intramuscular M_{SS} and M_{AS} tumors, arising from an inoculum of 1 million cells*

Tumor line	Age of solid intramuscular tumor (*days*)	No. mice inoculated with lung tissue	No. mice developing tumors	Ratio between columns 4/3
1	2	3	4	5
M_{SS}	4	5	0	0. 0
M_{SS}	7	8	2	0. 25
M_{SS}	12	8	4	0. 5
M_{AS_1}	3	4	0	0. 0
M_{AS_1}	6	8	8	1. 0
M_{AS_1}	9	8	8	1. 0

logic testing. The latter was performed by inoculating the minced and suspended lung tissue into C3H mice, as previously described in detail (*6*). The results of the inoculation tests are shown in table 2. In close agreement with the previous findings, lungs of M_{AS}-bearing mice transmitted the tumor earlier and more regularly than in the case of M_{SS}. The results of the histologic examination appeared paradoxical at first sight. With M_{SS}, tumor cells were seen in the lungs in the form of compact intravascular emboli, consisting of solid-tumor tissue, after 10 to 11 days (fig. 12). With M_{AS}, no tumor cells could be detected with certainty until 15 to 16 days, although foci of leukocytes, possibly surrounding isolated but not clearly identifiable tumor cells, were seen much earlier. After 15 to 16 days, metastatic tumor cells could be detected with certainty. Their distribution was quite different from M_{SS}, however; they were never seen as vascular emboli but in small foci of predominantly subpleural location, with loosely connected tumor cells in alveolar capillaries or intra-alveolarly (fig. 13). The connective tissue of the lung was diffusely infiltrated by leukocytes with both tumor sublines.

Although M_{AS} tumor cells were identified later than M_{SS}, their early detection was made very difficult by the type of distribution described. The histologic findings do not, therefore, invalidate the results of the biologic testing; the difference is most probably due to the fact that a very small number of tumor cells will readily show up on biologic testing, while histologic examination only detects a more advanced stage where the tumor-cell number has already reached a sizable level. Furthermore, individual tumor cells are difficult to identify on histologic sections and larger cell groups are necessary. This would make M_{SS} identifiable more easily and at an earlier stage by histologic examination of the lungs, in spite of the fact that loose cells of M_{AS} apparently reach the lung much earlier, as indicated by the biologic testing.

Subcutaneous Growth of M_{SS} and M_{AS}

To test whether the difference between the different tumor sublines with regard to intraperitoneal infiltration and the accompanying inflammatory reaction is also reflected when the cells grow in the solid form at another location, 10^6 M_{SS} and M_{AS} cells, respectively, were inoculated subcutaneously into the right flank of C3H mice. Previously it had been found that the 2 lines grow at the same rate after subcutaneous inoculation. Pairs of mice were killed daily during the first 6 days after inoculation and pieces of skin and the underlying subcutaneous tissue containing the inoculated tumor were fixed and studied histologically. No major difference could be found. Both sublines grew equally well and equally rapidly at this location. The majority of the tumor cells had a well-preserved and vital appearance. An inflammatory reaction of the type seen in and around the peritoneal implants of M_{AA} and M_{AS} could not be detected; inflammation was found only in the surroundings of necrotic areas and was then of the granular leukocytic type. There was only one detectable difference between M_{SS} and M_{AS}—the infiltrate of M_{AS} was looser and the tumor cells were more widely spread and generally more round than M_{SS} cells. In the latter, cells were more compactly arranged into solid-tumor tissue and were often spindle-shaped or polygonal. This difference was apparent as late as 6 days after inoculation (figs. 14 and 15). M_{SS} was also characterized by more frequent and more extensive necrosis than M_{AS}.

Discussion

Table 3 is a summary of the main results. In agreement with earlier findings, the original solid subline of the MC1M sarcoma, designated M_{SS}, was unable to grow in the peritoneal fluid, *i.e.*, it was not immediately convertible to the ascites form (A-form). In contrast, the ascites subline (M_{AA}) and the serially transferred solid subline developed from M_{AA} (M_{AS}) showed good growth in the free-cell form, in spite of 56–63 serial solid transfers in the latter case. This confirms, through a more extended observation period, the previous conclusion (*6*) that gradual transforma-

TABLE 3.—*Tabulation of the main results*

Tumor subline	Growth in peritoneal fluid	Signet-ring cells in peritoneal fluid	Growth in solid intra-abdominal tissues	Round-cell infiltration around peritoneal implants	Subcutaneous growth	Inflammation around subcutaneous implants	Necrosis in subcutaneous tumor	Metastatic cells in lung, determined by biologic testing	Histologic type of lung metastasis
M_{88}	−	−	+++	−	+++	−	Extensive	Early	Compact intravascular emboli
M_{410}	++	−	+++	−+					
M_{AA}	+++	−	+++	++					
M_{A8}		+	++	++	+++	−	Less extensive	Late	Loose subpleural groups of tumor cells

tion to A-form is stable and irreversible, even in the absence of exposure to the specific environment of the peritoneal fluid. One change was observed in M_{AS} as compared to M_{AA}, however. While the cytologic appearance of the solid tumors did not change during the serial transfer of M_{AS} to any appreciable extent, intraperitoneal testing revealed the presence of an increasing proportion of cells that reacted by excessive vacuolization when exposed to the environment of the peritoneal fluid, presenting the picture of the signet-ring cell. Provided that this is a degenerative phenomenon, this finding indicates some decrease in the degree of adaptation of this subline to the ascites form of growth, at least in a certain fraction of the tumor-cell population. This is also suggested by the increasing inflammatory reaction in the peritoneal fluid, paralleling the appearance of the signet-ring cells, since it was previously found that the intensity of inflammatory reaction in established ascites tumors usually parallels the process of tumor-cell death and degeneration (*10,11*). It seems that in subline M_{AS} a new cell type tends to replace gradually the original M_{AA} population, better fitted for growth in the solid form, perhaps, but reacting by excessive vacuolization when exposed to the environment of the peritoneal fluid. This new type does not represent a reverse change to the M_{SS} type, however, since M_{SS} cells never show a similar reaction after intraperitoneal inoculation. Such an interpretation is strengthened by the finding that both signet-ring cells and the inflammatory reaction disappear promptly and simultaneously if M_{AS} is permitted to grow in the peritoneal fluid for 4 or 5 uninterrupted serial transfers. This is presumably the result of adverse selection against the new signet-ring-forming cell type in the peritoneal fluid, favoring the dominance of the original M_{AA} type.

The behavior of subline M_{410} is interesting. This line was started with a comparatively small inoculum (5×10^5 cells). In agreement with previous findings, this reduction of the initial inoculum dose resulted in a decreased convertibility to A-form and the line is still not completely transformed, in spite of 23 serial exudate transfers. At present it shows a behavior intermediate between M_{SS} and M_{AS} with regard to the number of free tumor cells in the peritoneal fluid. It is possible that this is due to an as yet incomplete selection of a variant cell type that can grow in the peritoneal fluid only when inoculated in a sufficiently high concentration (*cf. 2*), the cell population thus being a mixture of 2 different cell types, analogous to M_{SS} and to M_{AA}, respectively. Alternatively, we may be dealing with the more or less pure culture of a variant distinctly different from both M_{SS} and M_{AA} with regard to growth potentialities in the peritoneal fluid. The very great variability between different individual mice inoculated with M_{410} with regard to the number of free tumor cells has often been noted previously with other sublines of the MC1M sarcoma, not yet completely converted to A-form, and often immediately prior to conversion.

The quantitative and qualitative differences in the ability of the 4 tumor sublines to produce solid-tumor implants in the peritoneal cavity

were quite unexpected, since they all grow equally well as solid tumors in the subcutaneous tissue, and those that were measured (M_{SS} and M_{AS}, ref. *12*) were found to have the same growth rate. At an early stage of our studies on the gradual transformation of solid tumors into ascites form (*1*), it was observed that the amount of gross and microscopic infiltration decreased as the process of conversion progressed. This was attributed, however, to a gradual increase of inoculum size with consequent decrease of survival time in the course of serial exudate transfer. In the present investigation this factor was eliminated, since the number of inoculated cells was kept constant and sublines of the same tumor, representing different developmental stages within the conversion process, were compared at successive intervals after inoculation. Under such conditions a most remarkable difference was found with regard to the extent and the morphology of implantation and the accompanying inflammatory reaction of the host. Growth in the solid intra-abdominal tissues showed an inverse relationship to free-cell multiplication in the peritoneal fluid. The lines not, or not entirely, converted to A-form (M_{SS} and M_{410}) infiltrated vigorously and extensively and there was an almost complete absence of any histologically detectable host reaction. On the other hand, the converted sublines (M_{AA} and M_{AS}) infiltrated to a much smaller extent, and their implants often contained tumor cells with signs of degeneration and poor vitality and were surrounded by a compact zone of round-cell infiltration.

The differences in the extent of solid-tumor infiltration were also reflected in the amount and appearance of the peritoneal fluid. The intensely infiltrating sublines produced strongly hemorrhagic ascites, disproportionately large in amount when related to its cell content. The converted and less infiltrative sublines were characterized by milky or slightly pink fluids with a much higher cell concentration, similar to other typical ascites tumors. It was suggested earlier that fluid accumulation in typical ascites tumors is related directly to the multiplication of free tumor cells and is not simply the result of mechanical obstruction caused by tumorous infiltration of lymphatics and small vessels (*5,9*). This was confirmed by the experiments of Straube *et al.* (*13*). The present results are in agreement with this concept, showing a different type of fluid accumulation in sublines where tissue infiltration is more intensive.

After inoculation of the original M_{SS} line, part of the cells adhere to the peritoneal surface, while others can be detected in the fluid for some time. The adherent cells soon begin to grow, invading the solid host tissues. The suspended cells cannot maintain themselves in the fluid and gradually disappear, either due to degeneration and lysis or to their eventual adherence to the peritoneal surface. When, on the other hand, M_{AA} or M_{AS} cells are inoculated, a certain proportion of them also adheres to the surface, while others remain in the fluid and continue to divide actively there. If the adhering cells had the ability to invade the solid tissues, as with M_{SS}, the result would be the proliferation of free tumor

cells in the peritoneal fluid, accompanied by large solid infiltrates. This is not the case, however, as mentioned above. In seeking an explanation, significance may be attached to the degeneration of M_{AA} and M_{AS} cells in the solid tissues and the concomitant inflammatory reaction. The causal relationships between these 2 events are not known; it is conceivable that an inflammatory reaction, directed specifically against M_{AA} and M_{AS} cells, causes their degeneration. It is equally possible, however, that inflammation is secondary to tumor-cell degeneration, which may occur for some other reason. It is noteworthy that neither cell degeneration nor an inflammatory response could be seen during growth in the subcutaneous space. This suggests that the phenomenon observed is not a matter of general host reaction against M_{AA} and M_{AS}, as contrasted to M_{SS} and M_{410} cells. Such a reaction is also made improbable by the fact that all 4 sublines used have been derived from the same original tumor, induced in the inbred C3H mouse strain and maintained by serial transplantation in the strain of origin or its F_1 hybrids. All 4 lines give a 1 histocompatibility-factor ratio when tested for progressive growth in $(A \times C3H)F_2$ hybrids, which indicates the absence of any major immunogenetic difference. The reaction in the peritoneal tissues against 2 of the sublines seems to be a local phenomenon, whether of primary importance or secondary to cell degeneration.

Whatever its mechanism, the fact remains that variant cells of the MC1M sarcoma that were concentrated by the selection process (*2*) occurring during gradual transformation into ascites form differ from the original cell type not only with regard to an increased capacity to grow in the peritoneal fluid but also in their greatly impaired ability to become implanted and grow subsequently in the solid tissues of the peritoneal cavity. The implication that the capacity to grow in the peritoneal fluid *versus* the capacity to grow in solid abdominal tissues are 2 dissociated characteristics is also indicated by the finding (G. Klein, unpublished data) that, while it is regularly possible to convert M_{SS} into M_{AA} by serial transplantation of tumor-cell-containing peritoneal *fluid*, it was never possible to achieve the same by serial intraperitoneal transfer of solid peritoneal implants. It would appear that different cells of the same original tumor may differ with regard to the "soil" they prefer for their growth, at least in the present case, where peritoneal fluid is contrasted with solid intra-abdominal tissues. Indirectly this lends support to the "soil hypothesis" constructed to explain the differential spread of tumor cells in the organism (*14*). The nature of the cellular variation responsible for differences in behavior in different "soils" is not known; it could be nutritional, metabolic, immunologic, etc. Whichever the case may be, at least in the present instance we are undoubtedly dealing with stable, irreversible, and hereditary characteristics, as shown by the history of M_{AS}.

The difference in the type and time of appearance of metastatic tumor cells in the lungs of mice bearing intramuscular M_{SS} and M_{AS} tumors, respectively, may be related to the reduced adhesiveness (*6*) and increased

mobility (*15*) of the M_{AS} cell variant. Adhesiveness has been shown to play an important role in determining the ability of different tumors to metastasize (*16*). The experiments on the sublines of the MC1M sarcoma show that even cells derived from the same original tumor may differ with regard to adhesiveness and the way in which they spread to the lungs. This indicates the possibility of producing, by appropriate methods of selective transfer, tumor sublines that differ in their ability to metastasize. So far, it has not been possible, however, to select a subline with increased metastasizing ability by serially transplanting lungs of M_{SS}-bearing animals through 12 transfer generations; nor did this lung subline show an increased ability to grow in the peritoneal fluid. This is not surprising in view of the finding (*2*) that the frequency of variant cells with the capacity to grow in the peritoneal fluid is very low in the M_{SS} population, of the probable order of 1 in 2.4×10^6 cells. Since serially transplanted lungs contain only a very small fraction of the M_{SS} cell population, the variant forms are probably excluded in most cases.

Finally, some consideration should be given to the question of whether the differences found between the various sublines of the MC1M sarcoma, differing in their capacity to grow in the peritoneal fluid, can be extrapolated to other neoplasms differing in their convertibility to A-form. Our preliminary experiments indicate that this is not necessarily so. The relationship between the growth of free tumor cells and the infiltration of tissues and the change of this relationship as a function of time are characteristics that apparently vary from tumor to tumor. Comparisons between different tumors mean very little, since each neoplasm represents only one particular stage in a series of developmental events. To study the correlates of the ascites transformation it is necessary to compare not different tumors, but different sublines of the same tumor, differing in convertibility. That such series might yield results analogous to the present findings is indicated by the previous observations about the conspicuous decrease of solid infiltration during the gradual conversion of 5 different neoplasms into A-form (*1*).

Summary

Four different sublines of the MC1M mouse sarcoma, representing different stages in the process of gradual conversion into ascites form (A-form), were compared with regard to the multiplication of free tumor cells in the peritoneal fluid and as to their capacity for infiltrative growth in various solid tissues of the abdominal cavity after intraperitoneal inoculation of a standard cell dose. Two sublines that do not possess the full capacity to grow in the peritoneal fluid exhibited vigorous and extensive infiltrative growth in the subperitoneal fat and connective tissue. In these sublines there were no signs of an inflammatory host reaction. In contrast, 2 sublines that grow readily in the ascites form showed only very superficial and scanty implants, consisting mostly of tumor cells with a degenerated appearance and lacking mitotic figures. Such areas were usually surrounded by a dense zone of lymphocytes. Thus there seems to

be an inverse relationship between the capacity of the 2 main cell types, derived from the same original tumor and differentiated by means of selective transfer, to grow in the peritoneal fluid and the peritoneal fat, respectively. This lends some support to the "soil hypothesis."

The 2 main solid sublines (M_{SS} and M_{AS}), differing in convertibility to A-form, also differed with regard to the time of appearance of tumor cells in the lungs after intramuscular implantation and the type of lung metastasis. M_{SS}, not convertible to A-form, produced compact intravascular emboli at a comparatively late stage. M_{AS}, derived from the ascites line, was characterized by the diffuse and extensive early spread of incohesive tumor cells through the lungs. Both M_{SS} and M_{AS} grew equally well in the subcutaneous space and no inflammatory reaction could be seen with either one. M_{AS} was characterized by a conspicuous looseness and lack of cohesiveness of the tumor cells, while M_{SS} grew as a compact tissue.

Comparisons between the ascites line (M_{AA}) and the solid line derived from it (M_{AS}) and maintained through 63 uninterrupted serial subcutaneous transfers have confirmed the stability and irreversibility of the conversion to A-form. M_{AS} still grows readily and immediately in the peritoneal fluid, and no reverse change to the M_{SS} type can be detected. The histopathologic appearance of the solid implants in the peritoneal fat were closely similar with M_{AS} and M_{AA}. The M_{AS} cells show an increasing tendency to develop signet-ring form after intraperitoneal inoculation, however, indicating the appearance of a new cell type. Concomitantly, the content of ascitic fluid in nontumorous cells shows an absolute increase. Both signet-ring cells and the excess of nontumorous cells can be brought to disappearance by 4 or 5 generations of intraperitoneal transfer of ascitic fluid.

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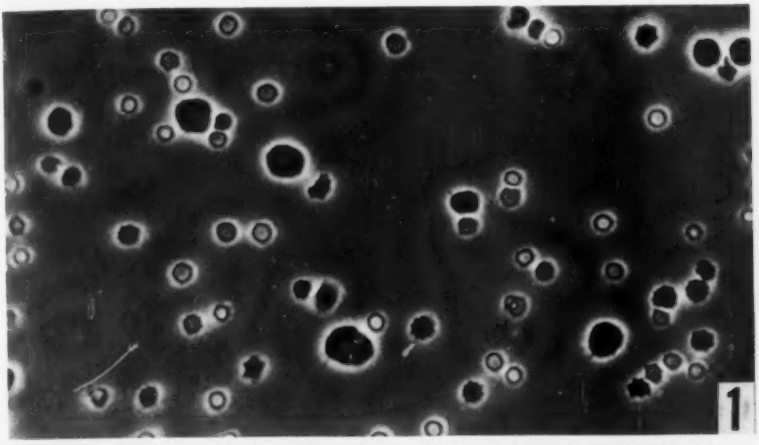
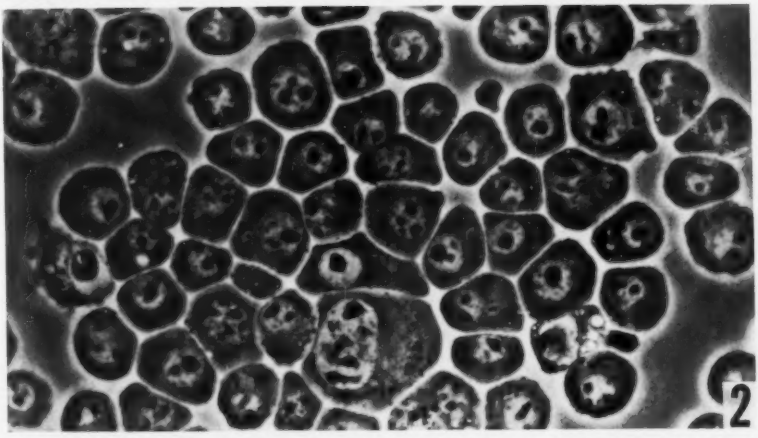
PLATE 16

FIGURE 1.—Peritoneal exudate from a C3H mouse, 10 days after inoculation with 10^6 M_{SS} cells. No tumor cells visible. The field contains erythrocytes and a few nontumorous nucleated cells. Phase contrast. × 900

FIGURE 2.—Ascites from a C3H mouse, 10 days after inoculation of 10^6 M_{AA} cells. Nearly pure culture of free tumor cells. Phase contrast. × 900

FIGURE 3.—Ascites from a C3H mouse, 10 days after inoculation of $10^6 M_{AS_{24}}$ cells. Free tumor cells dominate the picture, with frequent occurrence of signet-ring forms. Phase contrast. × 900





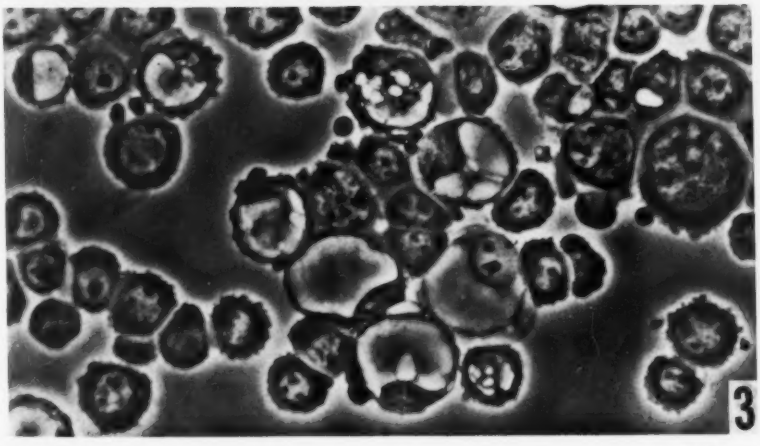


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PLATE 17

FIGURES 4 AND 5.—Low-power view of slides of randomly assembled abdominal-tissue fragments. Figure 4 from a mouse with M_{AA}, 12 days after intraperitoneal inoculation. *Arrows* indicate the sites of the very small tumor implants, hardly visible in the low magnification. Figure 5 from a mouse with M_{88}, 12 days after inoculation. Almost all tissue shown consists of tumor. The *dotted lines* surround the only areas free from tumor. × 4

FIGURE 6.—Early peritoneal implant of M_{AA}, 7 days after inoculation. Superficially, a coating of rather pale stained tumor cells with blurred contours; centrally, in the nodule, inflammatory cells (lymphocytes) with small dark nuclei. × 260

FIGURE 7.—Tumor infiltration of omental plica. M_{AA}, 6 days after inoculation. The tumor cells form a diffuse superficial coating. Centrally, in the plica, a dense lymphocytic infiltration (small, dark nuclei). × 130

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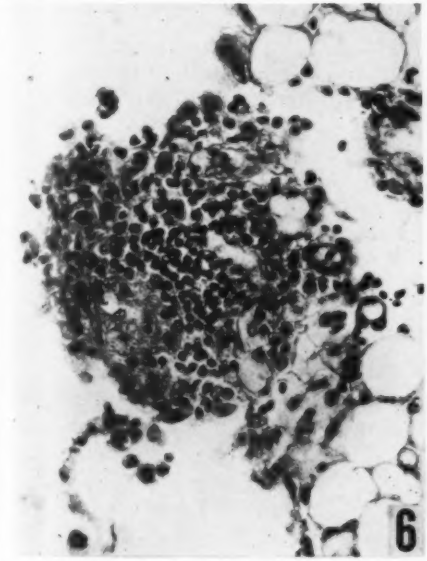
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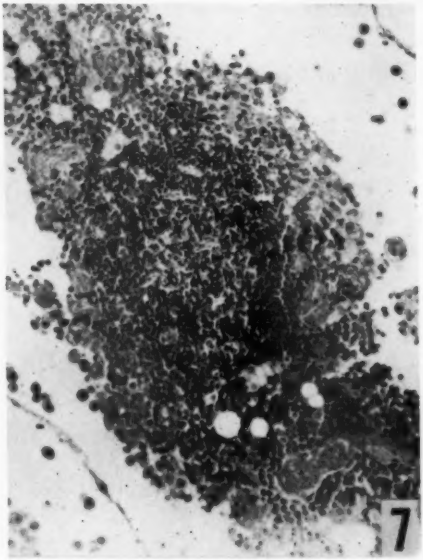
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PLATE 18

FIGURE 8.—Tumor infiltration of omental plica. $M_{AS_{56}}$, 4 days after inoculation. The pale, blurred tumor cells form a diffuse superficial coating. Centrally, a dense lymphocytic infiltration. × 220

FIGURE 9.—Small peritoneal implant of M_{AA}, 10 days after inoculation. The tumor cells have almost completely vanished and there is a very dense lymphocytic infiltration. × 260

FIGURE 10.—Early peritoneal implant of M_{SS}, 4 days after inoculation. The tumor tissue infiltrates deeply, surrounding the fat cells of the omentum. The tumor cells have a vital appearance with darkly stained nuclei and mitotic figures. No inflammatory infiltration (same as figure 6). × 260

FIGURE 11.—Detail of large omental implant of M_{410}, 10 days after inoculation. The tumor tissue surrounds sparse remnants of fat cells. The tumor cells have an appearance of high vitality, with well-stainable nuclei, visible nucleoli, and numerous mitotic figures. No inflammatory infiltration. × 220

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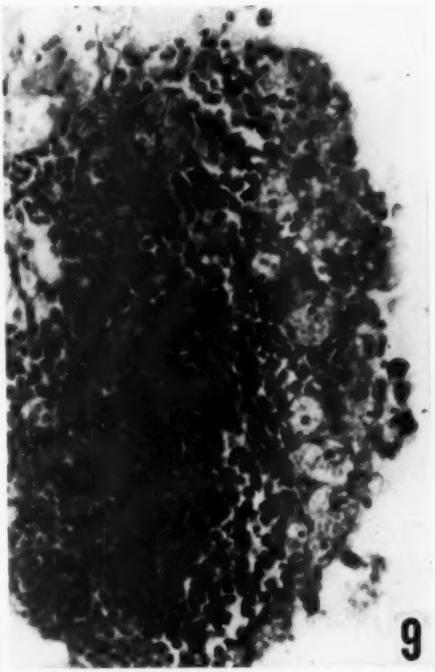
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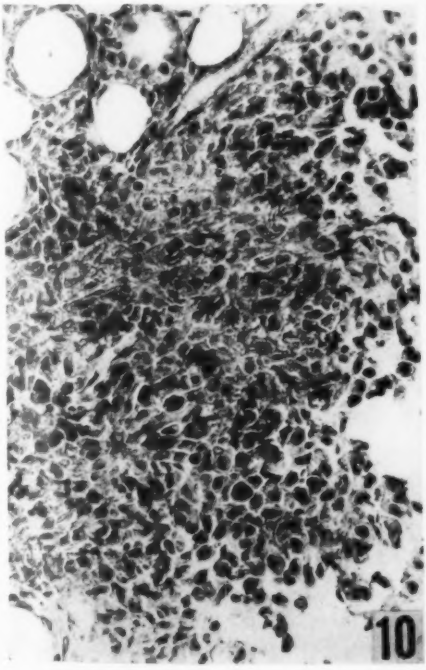
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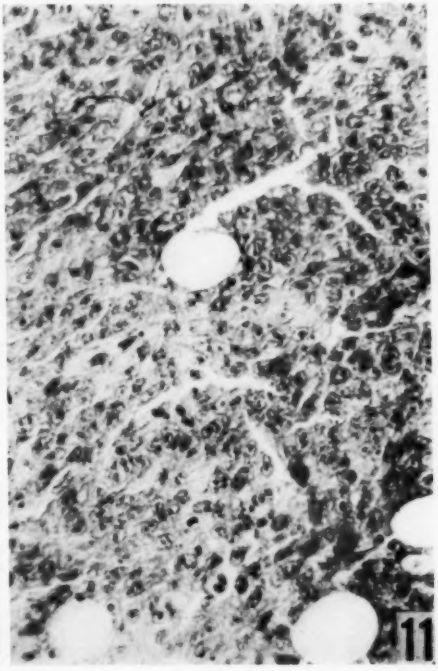
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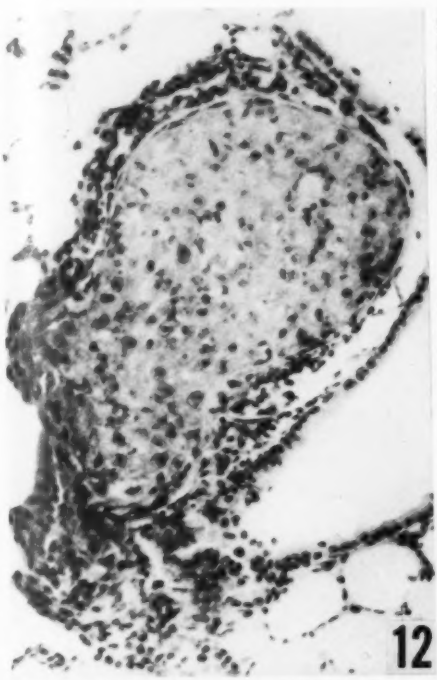
PLATE 19

FIGURE 12.—Pulmonary metastasis of M_{SS}, 23 days after intramuscular inoculation. Arteriolar embolus consisting of densely packed tumor cells. *Below*, adjacent to the embolus, the epithelial coating of a small bronchus. × 60

FIGURE 13.—Pulmonary metastasis of M_{AS}, 15 days after intramuscular inoculation. The subpleurally located metastasis consists of diffusely spread tumor cells located individually or in groups of a few cells in the alveolar capillaries. × 60

FIGURE 14.—Border zone of subcutaneous infiltrate of $M_{AS_{63}}$, 5 days after inoculation. The tumor cells are diffusely spread in the meshwork of connective-tissue fibers. × 120

FIGURE 15.—Border zone of subcutaneous infiltrate of M_{SS}, 6 days after inoculation. The infiltrate consists of closely packed cells and the borderline against the subcutaneous tissue is sharp. × 120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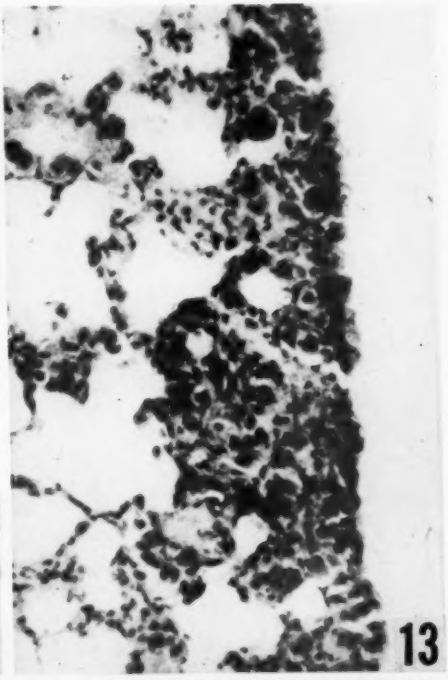
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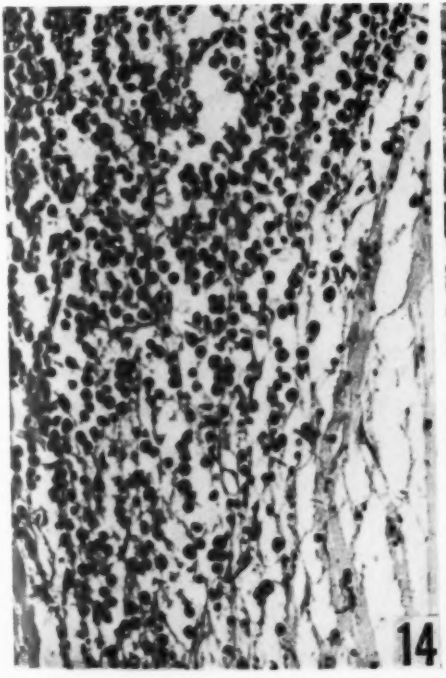
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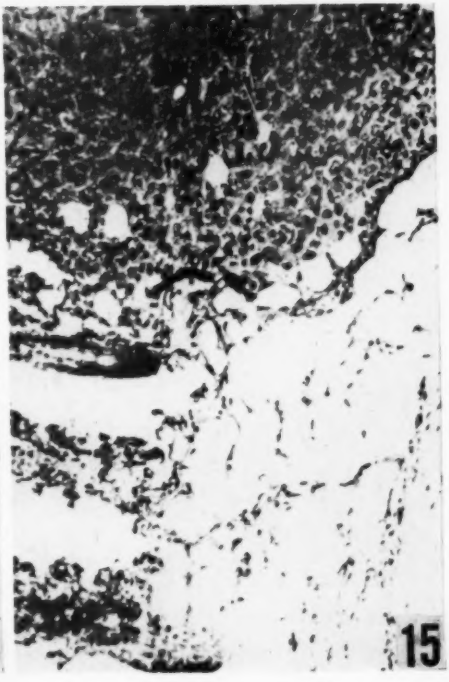
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Combination Chemotherapy: Synergism between a Possible Folic Acid Antagonist and Ethionine on a "Solid" Tumor [1,2]

DANIEL M. SHAPIRO *and* RUTH A. FUGMANN,[3,4]
Department of Surgery, College of Physicians and Surgeons, Columbia University, New York, New York

The efficacy of combinations of antimetabolites in cancer chemotherapy the subject of continuing investigations in this laboratory (*1–5*). The operational hypothesis is that chemical combinations can be developed to affect tumor growth adversely, to the point of eradication, without producing either serious host toxicity or drug resistance. Wherever feasible, the concept of sequential blockade, as defined by Potter (*6*), is employed in the selection of antimetabolite combinations. For example, an effective folic acid antagonist should inhibit 1-carbon transfer and, hence, decrease cellular methionine synthesis due to interference with homocysteine conversion to methionine. The addition of a methionine antagonist should then, due to this cellular diminution in methionine level, be more effective.

Skipper *et al.* (*7*) have noted such synergism between amethopterin and ethionine against mouse leukemia. The compound 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine, hereafter designated as 50-197, has been thought to act like a folic acid antagonist (*8*). It was, therefore, considered feasible to look for a possible sequential-blockade effect by giving 50-197 in combination with ethionine against a "solid" tumor, the mouse mammary adenocarcinoma 755.

Materials and Methods

Strain C57BL mice, 2 to 3 months old and weighing from 18 to 25 gm., were housed in plastic cages in an air-conditioned, constant-temperature room (74° F.) and received Rockland pellets and water *ad libitum* throughout the experiment. The neoplasm employed was the Bagg-Jacksen mammary adenocarcinoma 755, implanted into the axillary region by the usual trocar technique. There were 20 tumor-bearing animals in each experimental group.

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TABLE 1.—*Effect of ethionine on mammary adenocarcinoma 755 in C57BL mice*

Expt. no. and sex	Group	Dose (*mg./kg.*)	No. mice injected	Day injection begun following transplantation	Avg. tumor wt. ± S.E.	No. dead / Total	Host wt. change (*percent*)
240 ♀	Control	—	14	3	408 ± 98	0/20	+8
	Ethionine	150	14	3	263 ± 102	3/19	+2
	Ethionine	150*	8	3	280 ± 88	0/20	+6
274 ♀	Control	—	6	1	331 ± 46	0/20	+1
	Ethionine	100*	6	1	197 ± 30	0/20	−5
	Ethionine	150*	6	1	265 ± 31	0/21	−6
337 ♀	Control	—	11	5	351 ± 53	0/19	+2
	Ethionine	100	11	5	132 ± 20	1/20	−7
307 ♂	Control	—	9	1	2198 ± 330	5/20	+13
	Ethionine	200*	9	1	1825 ± 292	5/20	+9
239 ♂	Control	—	14	3	564 ± 109	1/20	+10
	Ethionine	150	14	3	711 ± 99	4/20	−1
	Ethionine	150*	8	3	545 ± 134	1/20	+10
233 ♂	Control	—	13	4	1063 ± 142	6/20	+6
	Ethionine	100	13	4	1000 ± 120	4/20	+1
	Ethionine	100†	13	4	950 ± 156	2/19	−6
	Ethionine	100	7	4	1129 ± 182	5/20	+7
	Ethionine	100*†	7	4	939 ± 159	5/20	+3
273 ♂	Control	—	6	1	324 ± 43	0/19	+7
	Ethionine	100	6	1	224 ± 32	0/20	+1
	Ethionine	150	6	1	282 ± 43	1/20	+3

*Injected intraperitoneally every other day; unstarred groups injected daily.
†In slightly basic solution.

The compound 50-197, in slightly acid solution, was freshly made every 3 days and stored in the refrigerator, while ethionine was dissolved daily in saline by shaking. Both compounds were administered intraperitoneally. Ethionine was injected a half hour prior to 50-197 in all combination experiments, except in those where 50-197 was administered twice daily; in the latter, ethionine was given simultaneously with the second daily injection of 50-197.

In order to ascertain the presence or absence of antineoplastic effect, the tumors were removed the day after the last injection and wet weights were determined to the nearest milligram. Results were considered to have a chance variation of 1 percent or less when

$$\frac{m_1 - m_2}{(m_1)^2 + (m_2)^2} = 2.5 \text{ or greater,}$$

where m_1 and m_2 are the average tumor weights of the 2 groups being compared.

Results

Experiments were conducted with ethionine alone, at levels (100 mg./kg. to 200 mg./kg.) which have been reported to cause carcinostasis of the 755 tumor (7). However, we considered ethionine to be without significant carcinostatic effect (table 1), since in only 1 of these 7 experiments (expt. #337) did this compound cause statistically significant inhibition of adenocarcinoma 755.

The effect of 50-197 by itself on the tumor was investigated by employing 3 different levels of the compound (table 2). At a 2 mg. per kg. dose administered for 3 consecutive days and every other day thereafter, 50-197 caused statistically significant tumor inhibition in 5 out of 8 experiments (63%) in male mice, and in only 2 out of 8 experiments (25%) in female mice. At both a 1 mg. per kg. daily dose and a 0.3 mg. per kg. twice-daily dose, 50-197 showed no inhibitory effect on the tumor in mice of either sex.

The effect of addition of ethionine to 50-197 upon the growth of adenocarcinoma 755 was studied by combining ethionine, at a dose of 65 mg. per kg. daily, with each of the above doses of 50-197. When ethionine was combined with the 2 mg. per kg. dose of 50-197 (table 2), significant carcinostasis was observed in 4 out of 5 experiments (80%) in male animals, and in 4 out of 7 experiments (57%) in female animals. The combination of ethionine with the 1 mg. per kg. dose of 50-197 caused significant carcinostasis in all experiments (100%) with male mice and in 3 out of 4 experiments (75%) with female mice. Addition of ethionine to the 0.3 mg. per kg. twice-daily dose of 50-197 evidenced significant tumor inhibition in 5 out of 6 experiments (83%) in male animals but showed no anti-tumor effect (0%) in female animals.

Discussion

Ethionine, which is noncarcinostatic by itself, appears to potentiate the very weak carcinostatic action of 50-197. For example, by adding

TABLE 2.—*Effect of 50-197* alone and in combination with ethionine on adenocarcinoma 755 in C57BL mice*

Agent	Dose†‡ (*mg./kg.*)	Avg. tumor growth§ (*percent*)	Avg. host mortality (*percent*)	Avg. host wt. change (*percent*)	No. experiments		
					Total	No. significant	Percent significant
			Male				
50-197 alone	2	57	1	−1	8	5	63
	1	70	0	+1	3	0	0
	0.3 × 2	80	2	+5	6	0	0
50-197 + ethionine (in combination)	2	42	9	−3	5	4	80
	1	38	0	−6	3	3	100
	0.3 × 2‖	54	9	−3	6	5	83
			Female				
50-197 alone	2	70	6	+1	8	2	25
	1	69	1	+4	4	0	0
	0.3 × 2	132	10	+6	3	0	0
50-197 + ethionine (in combination)	2	38	10	−4	7	4	57
	1	43	5	−2	4	3	75
	0.3 × 2‖	103	16	+1	3	0	0

*2,4-Diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine.

†Doses of 50-197: 2 mg./kg. administered intraperitoneally daily for 3 consecutive days and every other day thereafter; 1 mg./kg. administered intraperitoneally daily; 0.3 mg. × 2 administered intraperitoneally twice daily at a 4-hour interval.

‡Ethionine administered intraperitoneally at 65 mg./kg.

§Saline control taken as 100 percent tumor growth.

‖Ethionine administered with second daily dose of 50-197.

ethionine to the noncarcinostatic 1 mg. per kg. dose of 50-197, the consistency of observed carcinostasis (table 2, No. Significant Experiments) was increased from 0 to 100 percent in males and from 0 to 75 percent in females. Since the combined effect of the 2 agents is greater than a simple algebraic summation of their individual carcinostatic activities, the result was considered to indicate synergism.

Although there is evidence of weight loss in these experiments, it is not felt that the degree of weight loss recorded here is in any way responsible for the inhibitory effect on the tumor. Unpublished observations in this and another laboratory demonstrate that growth of adenocarcinoma 755 is not significantly affected by host weight loss up to 10 percent (*9*).

It is apparent that there is a sex difference in tumor response to these drugs. In all experiments there was slightly greater anti-tumor effect with the male host mouse. However, with the combination of ethionine and the 0.3 mg. per kg. twice-daily dose of 50-197, the sex difference was marked (tables 3 and 4). This dosage schedule was entirely without antineoplastic effect in female animals but produced statistically significant anti-tumor results 83 percent of the time in male animals. It is of interest, however, that this sex difference can be lessened by the choice

TABLE 3.—*Effect of combination chemotherapy with 50-197* at a* low fractionated dose *(0.3 mg./kg.) and ethionine (65 mg./kg.) on mammary adenocarcinoma 755 in* male *C57BL mice*

Expt. no.	Group	No. mice injected†	Day injection begun following transplantation	Avg. tumor wt. ± S.E.	No. dead / Total	Host wt. change (*percent*)
177	Control	14	3	1082±194	0/20	+12
	50-197	14	3	881±130	0/21	+5
	50-197+eth.	14	3	517±117	2/21	−1
221	Control	11	6	845±125	0/20	+5
	50-197	11	6	607± 76	0/20	+6
	Ethionine	11	6	771±165	0/20	+4
	50-197+eth.	11	6	363± 53	2/20	−4
277	Control	13	7	1949±233	3/20	+18
	50-197	13	7	1500±157	0/19	+3
	Ethionine	13	7	1881±236	2/19	+11
	50-197+eth.	13	7	1116±121	3/20	−6
283	Control	13	5	1390±182	1/20	+2
	50-197	13	5	1331±182	0/19	−2
	50-197+eth.	13	5	1081±153	2/20	−7
291	Control	15	5	1667±246	0/20	+14
	50-197	15	5	1469±162	1/20	+10
	Ethionine	15	5	1889±211	2/20	+16
	50-197+eth.	15	5	761±136	1/20	−6
293	Control	14	5	1857±217	0/19	+16
	50-197	14	5	1223±185	1/19	+8
	50-197+eth.	14	5	1021±143	1/20	+6

*2,4-Diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine.

†50-197 was administered intraperitoneally at a dose of 0.3 mg./kg. twice daily, 4 hours apart; ethionine was given intraperitoneally once daily with the second injection of 50-197, at a dose of 65 mg./kg.

TABLE 4.—*Effect of combination chemotherapy with 50-197* at a* low fractionated dose *(0.3 mg./kg.) and ethionine (65 mg./kg.) on mammary adenocarcinoma 755 in* female *C57BL mice*

Expt. no.	Group	No. mice injected†	Day injection begun following transplantation	Avg. tumor wt. ± S.E.	No. dead / Total	Host wt. change (*percent*)
210	Control	20	3	907±233	0/20	+7
	50-197	20	3	823±120	1/21	+1
	Ethionine	20	3	629±120	3/20	+4
	50-197+eth.	20	3	603±115	3/21	+1
248	Control	15	4	467±122	2/19	+1
	50-197	15	4	753±119	4/20	+5
	Ethionine	15	4	413± 91	2/20	+8
	50-197+eth.	15	4	664±115	3/19	−1
286	Control	13	5	864±216	4/20	+10
	50-197	13	5	1252±215	1/20	+10
	Ethionine	13	5	730±121	1/20	+5
	50-197+eth.	13	5	894±136	4/20	+3

*2,4-Diamino-5-(3',4'-dichlorophenyl)-6-methylpyrimidine.

†50-197 was administered intraperitoneally at a dose of 0.3 mg./kg. twice daily, 4 hours apart; ethionine was given intraperitoneally once daily with the second injection of 50-197, at a dose of 65 mg./kg.

of a proper dose combination. The marked sex difference in tumor response to the combination of ethionine and 0.3 mg. per kg. of 50-197 was nullified to a considerable degree when the latter was given at levels of 2 mg. per kg. or 1 mg. per kg. (table 2).

Sex differences in drug response are not unusual (*10–12*). In particular, there have been numerous reports of sex differences in response to folic acid and its analogs (*13,14*). Similar differences in response have been documented with ethionine (*15,16*), where the females suffer greater liver damage than do males. Although a few theoretical explanations have been advanced, these sex differences are not yet understood and the mechanisms through which these variations are mediated have yet to be elucidated.

Summary

2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine (50-197), a possible folic acid antagonist, when administered alone exerted a weak carcinostatic effect against Bagg-Jacksen mammary adenocarcinoma 755 in C57BL mice.

Ethionine alone had no carcinostatic action against this tumor.

The combination of these 2 drugs under certain conditions showed a synergistic anti-tumor effect.

A sex difference in tumor response, which was dependent on dosage, was noted.

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Host Influence on Homoplastic Skin Grafts in Inbred Mice [1,2,3]

ANNABEL G. LIEBELT,[4] *Departments of Anatomy, Baylor University College of Medicine, Texas Medical Center, Houston, Texas, and University of Illinois College of Medicine, Chicago, Illinois*

The almost universal failure of skin homografts in man has led to laboratory investigation to ascertain the genetic and immunologic requirements of such grafts. Successful homografting in man has been accomplished only between monozygotic twins (*1*) and in cases in which the recipient has congenital agammaglobulinemia (*2*). Main and Prehn (*3*) obtained prolonged survival of homografts between inbred strains of mice by giving lethal doses of X irradiation, which were followed by protective intravenous injection of bone marrow from F_1 hybrids. Medawar (*4,5*) explained the homograft reaction in rabbits on the basis of actively acquired immunity to the foreign skin. Recently, with a sensitive serologic method (*6*), antibodies to skin homografts have been demonstrated.

With inbred strains of mice, success or failure of normal as well as tumor-tissue homografts depends primarily on the presence or absence of similar dominant "histocompatibility" genes in both donor and host. Using F_1 hybrid hosts, Prehn (*7*) grafted skin from one parental strain and then implanted a tumor from the other parental strain into the skin graft. Since the tumor grafts "took," he concluded that the incompatible tissue at the tumor-implantation site did not influence the fate of the transplanted tumor.

The mechanisms involved in the transplantation of normal as well as neoplastic tissue have recently attracted the efforts of many investigators. Attempts have been made to cross genetic barriers by varying the site of transplantation or by depressing the immune response of the host.

The experiments reported here were designed to investigate further the importance of the genetic control of humoral factors in the grafting of tissues in inbred mice.

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[3] Presented in part in Proceedings of the American Association for Cancer Research 2: 129–130, 1956 (abstract).

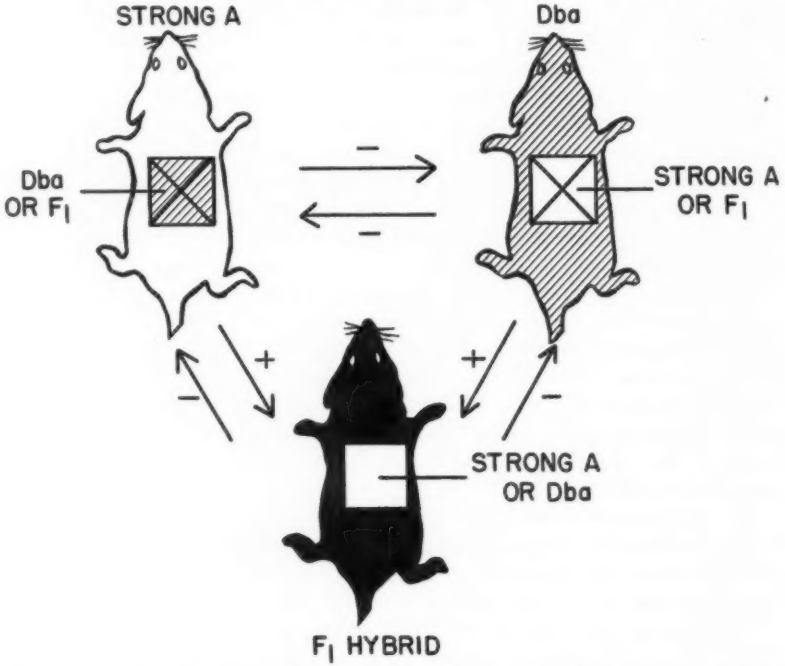
[4] *Present address:* Baylor University College of Medicine.

Materials and Methods

Skin grafts were made using strain A mice, DBA/2 mice, and black[5] F_1 hybrids, obtained by crossing these 2 parental strains; all mice were bred in this laboratory.

After clipping the hair of both donor and host, a full-thickness piece of skin, usually 2.5 × 2.5 cm., was excised from the inguinal region of the donor mouse. The skin remained in saline while the recipient was being anesthetized and prepared. The recipient site, on the dorsum of the mouse, was approximately the same size as the graft. Bleeding was minimal. The bed and graft were kept moist throughout the procedure. After the graft had been applied, many interrupted sutures of surgical silk #3–0 were made to approximate the skin edges. Sterile precautions were not observed and no dressings were applied.

All mice were housed individually following surgery. Observations were made daily. The criteria for viability or breakdown of the grafted



TEXT-FIGURE 1.—Diagram of skin grafts between incompatible and compatible mice. The 2 inbred strains were A (*white*) and DBA (*hatched*). The black F_1 hybrid is indicated by *solid black*. Unsuccessful grafting is indicated by (−) and the resultant slough by (X). Successful grafting from either parental strain into the F_1 hybrid is indicated by (+).

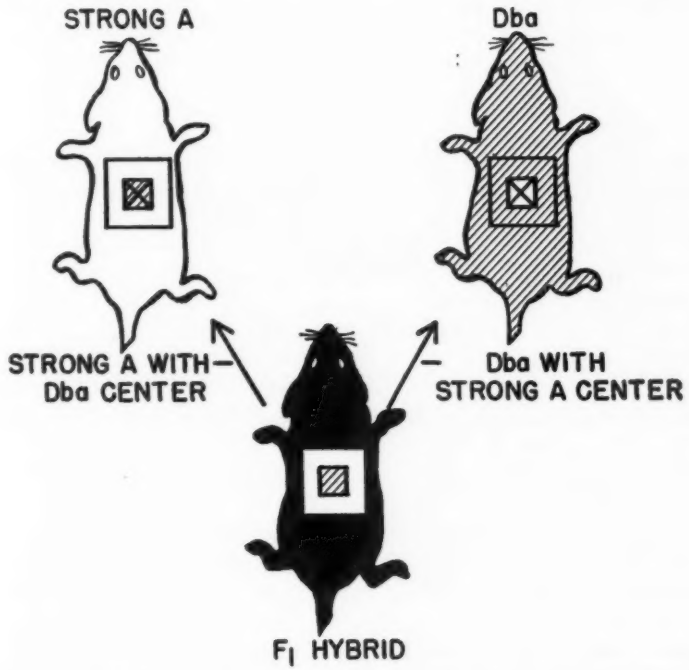
[5] The strain A mice used were inbred in this laboratory for 30 generations prior to use in these experiments. In other laboratories similar F_1 crosses are brown, but in this laboratory they are black. The *b* gene may have mutated to a *B*. Regardless of the explanation, the A mice used are homozygous, and both normal and neoplastic tissues are readily grafted within the strain.

skin were: color of the graft, texture, amount of desquamation and contraction, and the eventual restoration of the hair of the graft, or ultimate scabbing and sloughing.

Text-figure 1 diagrams the experimental plan, with both homografts[6] and isografts. Nineteen homografts were made between the A and DBA strains and from the F_1 hybrids into strain A mice.

Isografts were made from either of the parental strains into the F_1 hybrids. In 6 instances, after the graft had remained in the F_1 hybrid host for periods varying from 81 to 285 days, it was excised; one half was then regrafted to another F_1 hybrid and the other half returned to a mouse of the donor parental stock.

Another group of F_1 hybrid mice, after having received an isograft from one parental strain, then received within this isograft another "center" graft, usually 1.0 × 1.0 cm., from the second parental strain. This is diagrammed in text-figure 2. The second graft was made 25 to 332 days after the first. Thus, skin grafts from genetically different



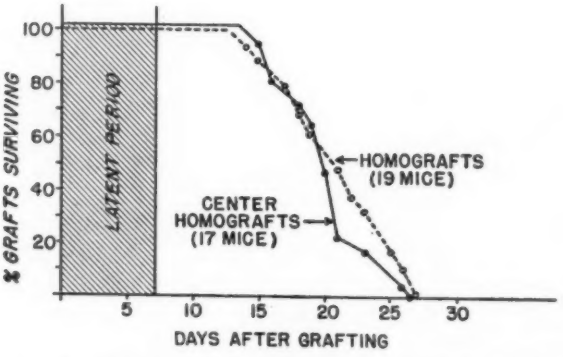
TEXT-FIGURE 2.—Concentric skin grafts from 2 genetically different parent strains, strains A (*white*) and DBA (*hatched*), in F_1 hybrid (*solid black*). Upon regrafting the larger graft, with its genetically different center, back to a donor strain, the center skin sloughed, as indicated by (X).

[6] *Homograft* refers to a graft made between mice of different inbred strains of different genotypes. *Isograft* refers to a graft, either between mice of the same inbred strain which are of the same genotype or between a donor of either parent strain and its F_1 hybrid offspring.

mice were made one within the other, concentrically. Seventeen of these isografts with their "center" grafts were returned to a mouse of the donor stock; *i.e.*, a strain A graft with a DBA center was regrafted to a strain A mouse.

Results and Discussion

The 19 skin homografts are represented in text-figure 3 and follow the same pattern of breakdown and final slough as other investigators have reported. There was a latent period of 4 to 11 days, averaging a week, during which the homograft appeared living and identical with an isograft. The first indications of breakdown were graft discoloration, loss of pliability, and weakening and separation of skin edges (fig. 1). Histologically, there was desquamation of superficial cells, thickening of the epidermis, and invasion of the dermis by leukocytes. Subsequently, the graft became hard, lumpy, and contracted. The scab sloughed within 2 to 3 weeks after initial grafting, leaving a small area which was re-epithelialized by the host.



TEXT-FIGURE 3.—Comparison of survival of homografts made between mice of strains A and DBA, or from F_1 to A mice, and survival of "center" homografts. *Note* similarity in survival times of foreign skin, whether the graft is of the usual type or one contained within a graft compatible with the recipient parent stock.

All isografts were completely healed and alive within 2 to 3 weeks (fig. 2). Eleven of 12 grafts made from 6 donors were successfully retransplanted to other F_1 hybrid hosts or to mice of the donor strains. All these grafts remained alive for 6 to 12 months, at which time the mice were killed. Thus, the parent tissue grafted to the F_1 hybrid and then regrafted to mice of the donor strain remained compatible with the latter.

In the F_1 hybrid host, grafting of tissues which were incompatible with each other (fig. 3) was successful in concentric positions. Histologic sections of the union between the "center" graft and the larger first graft, as well as between the larger graft and the F_1 hybrid host tissue, showed continuation between grafts, or host tissue and graft, of

the dermal elements, including blood vessels. There is no local tissue factor in incompatible tissues which prevents grafting them concentrically, as long as the host is compatible with both.

Seventeen isografts with "center" grafts from genetically different mice (8 strain A with DBA "centers," 2 DBA with A "centers," and 7 parental-strain grafts with F_1 hybrid "centers") were returned after 3 to 8 weeks to mice of the original donor strain. All the larger grafts which were compatible with the new hosts were alive after 2 to 3 weeks. All 17 "centers," as seen in text-figure 3, appeared as successful grafts for a latent period of approximately a week, following which signs of homograft breakdown appeared (fig. 4). The survival time and history of "center" homografts followed the same pattern as the usual homografts between strains.

Donor tissues became vascularized and survived permanently only in hosts which contained similar histocompatibility genes. This was true when genetically different tissues were grafted in concentric positions in a host histocompatible with both. When returned to a host compatible with one and not the other of the concentric tissues, breakdown occurred in the naturally incompatible tissue, indicating that it had retained its foreign nature.

Summary

Skin grafts from 2 genetically incompatible inbred strains of mice may be made successfully one within the other into F_1 hybrid hosts genetically compatible to both. Upon regrafting of the concentric, united grafts into a host compatible with only one of the grafts, breakdown of the incompatible one occurred, although it was thoroughly united to and even surrounded by the compatible graft. The grafted skin was altered by neither the F_1 hybrid host nor the concentric incompatible skin. After introduction into a new, genetically incompatible host the usual response to foreign tissue occurred.

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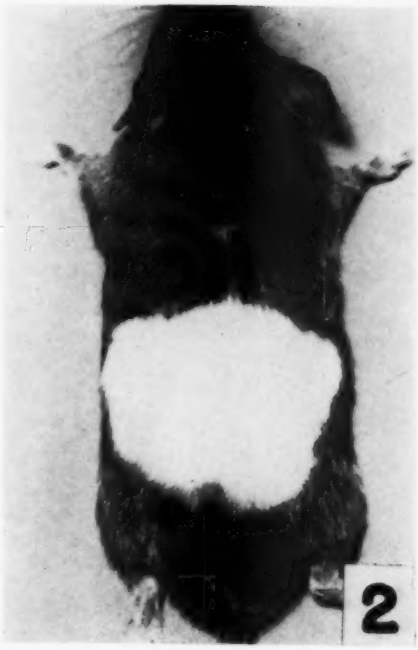
PLATE 20

FIGURE 1.—First signs of breakdown in a homograft of the usual type 8 days after grafting. Graft is dry and elevated from bed.

FIGURE 2.—Isograft from strain A mouse into F_1 hybrid 4 weeks after grafting. *Note* survival of graft.

FIGURE 3.—DBA "center" 51 days after grafting into a strain A graft (skins from 2 incompatible strains) in an F_1 hybrid host. *Note* abundant hair growth on both grafts. Two genetically incompatible skins unite as compatible tissues within a host compatible with each.

FIGURE 4.—Seventeen days after regrafting a large strain A graft with smaller DBA "center" (same type of graft shown in fig. 3 in an F_1 hybrid host) to a strain A host. *Note* homograft breakdown of "center" and simultaneous "take" of larger compatible graft.



Liebelt

407622—57——8

The Electron Microscopy of Tobacco Smoke [1]

H. KAHLER *and* BOLIVAR J. LLOYD, JR., *National Cancer Institute,*[2] *Bethesda, Maryland*

The large number of recent investigations on the chemistry of tobacco and tobacco smoke suggested that an electron-microscopic study of tobacco smoke would be of interest. For this purpose the smoke from cigars and cigarettes was studied, since the chemistry of the tobacco, as well as its physical structure, is different in each case.

Materials and Methods

Popular brands of cigarettes, with and without filter tips, and cigars with a long-leaf filler were used as the source of tobacco smoke. These were held in glass holders made especially to give a leak-proof seal. Standard puffs (*1*) of 35 ml. volume and 2 seconds' duration, repeated once a minute, were taken by a smoking machine. Two dilutions were used. When one puff, or 35 ml. of smoke, passed directly into the electrostatic precipitator the dilution was 1:15. When it passed through an intervening 7-liter flask the dilution was 1:200. The electrostatic precipitator (Mine Safety Appliance) was operated with a double-length collecting tube. The formvar-coated microscope grids were mounted on metal slides, which in turn were placed in the precipitator tube directly below the axial-wire electrode.

The precipitator was operated, usually for a period of 1 minute, at a field strength of 5000 volts per cm. The wire electrode was at a negative potential.

After the smoke particles were deposited on the grids they were removed, air dried and shadowed with a light coating of chromium at an angle with a tangent equal to 0.2, and examined in the electron microscope.

Results

Figure 1 is a fairly representative photograph of cigarette smoke containing a large number of particles. These particles have very low photographic contrast and cast very little shadow. The largest ones

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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show a pattern of a denser network than the surrounding material. At higher magnifications the network can be seen in some of the intermediate-size particles. In this view the particles range from a submicroscopic size of 18 mμ to 1.6 μ, with a weighted average diameter of 1/2 μ.

Some of the types of particles seen in cigar smoke are shown in figure 2. In contrast to cigarette smoke there are numerous fine particles below 0.1 μ arranged about the large central droplets.

A photograph illustrating the complex character of a large cigarette-smoke droplet is shown in figure 3. Internally, this structure resembles the large central droplet of figure 2; its general character suggests an emulsion. Figures 4, 5, and 6 are further illustrations of cigar-smoke patterns.

A microscope grid with formvar coating facing the combustion end of a cigarette was inserted into a slit cut across the width of the cigarette, 2 cm. from the mouth end. The cigarette was smoked by mouth in a normal fashion to half length. The screen was then removed for examination of the smoke collected on the formvar. Figure 7 indicates a heavy deposit of particles somewhat resembling in morphology the pattern in figure 1.

Inhaled-Exhaled Cigarette Smoke

A filter-tipped cigarette was smoked normally to half length. A 2-second puff was then inhaled deeply and held in the lungs for 5 seconds. It was then exhaled into the electrostatic precipitator and deposited on screens in the usual manner. Figure 8 illustrates a frequently occurring type of pattern for this smoke. The most noticeable feature of these particles, as compared to noninhaled cigarette smoke, is the greater number of peripheral particles surrounding the central large droplet. The 3-dimensional character of the central droplet, as shown by the long shadow, is a common type in all tobacco smokes so far examined, presumably occurring when the dried shell of the particle has sufficient rigidity to prevent the collapse of the particle on complete dessication.

It is evident that inhaled smoke particles must pass through a region of high humidity and that the tar droplets may accumulate an aqueous layer of unknown morphology.

Discussion

From this series of photographs it can be seen that the greater part of the smoke is in the form of liquid droplets. Usually particles casting long shadows, having high electron-scattering power, and of arbitrary shape, signifying solid particles rather than liquids, are surrounded by material that suggests liquid residues. The solid particles, being the first to condense in the cooler zone immediately ahead of the 800° C. (*2*) combustion zone, have a high probability of being coated with liquid materials which condense later at lower temperatures. We conclude that cigar smoke shows this characteristic to a greater extent than cigarette smoke.

The droplets have a heterogeneous chemical constitution, as judged by the fact that while part of the material volatilizes at room temperature, nonvolatile material remains as a residue—giving the electron-microscopic image.

If all the smoke (20 mg.) (*3*) from one cigarette is assumed to be made up of particles 0.4 to 0.6 μ in diameter (*4*) and a factor of 1/4 used for alveolar deposition (*5,6*), it can be calculated that this would coat about 1/100 percent of the alveolar surface of an area 7×10^5 cm.^{2} (*7*).

Summary

1) Electron micrographs of cigar and cigarette smoke, electrically precipitated, illustrate a variety of forms in which the smoke condenses and dries.

2) The dried specimens, essentially the solid residues of the smoke, consist of flattened, rounded forms—often with enclosed networks, solid particulates surrounded by amorphous coatings, and complex structures, which suggest an internal emulsion before drying.

3) The patterns formed from cigar smoke have a higher ratio of particulate to tarry material than cigarette smoke.

4) Usually the cigar-smoke pattern shows a central body surrounded by a peripheral ring of particles 1/10 to 1/100 its diameter.

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PLATE 21

FIGURE 1.—Cigarette smoke. Third puff. Diluted 15 times. × 18,500

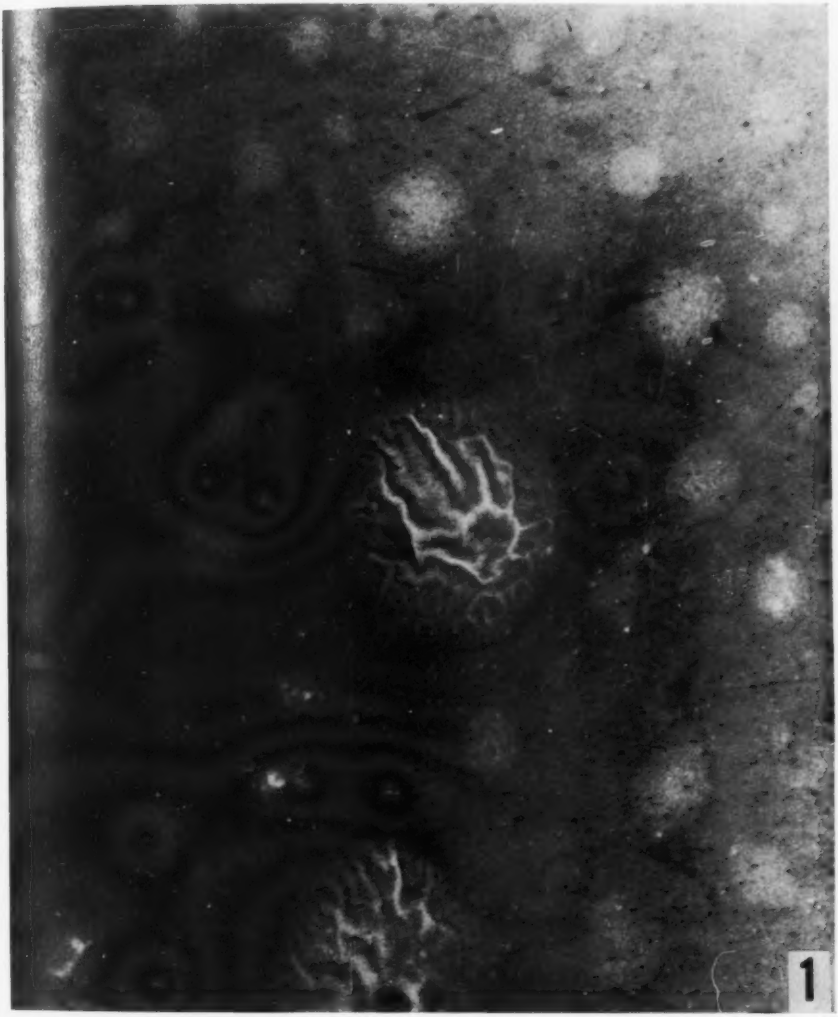
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PLATE 22

FIGURE 2.—Cigar smoke. Diluted 15 times. × 18,500

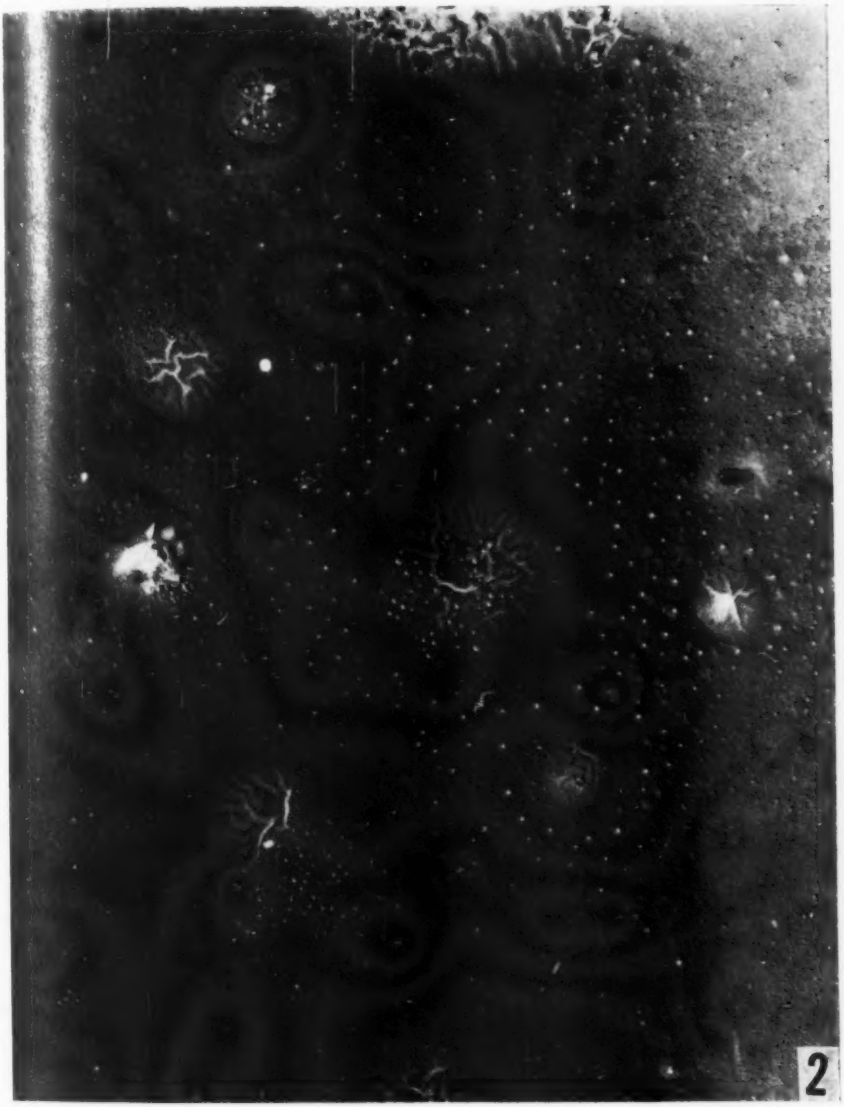

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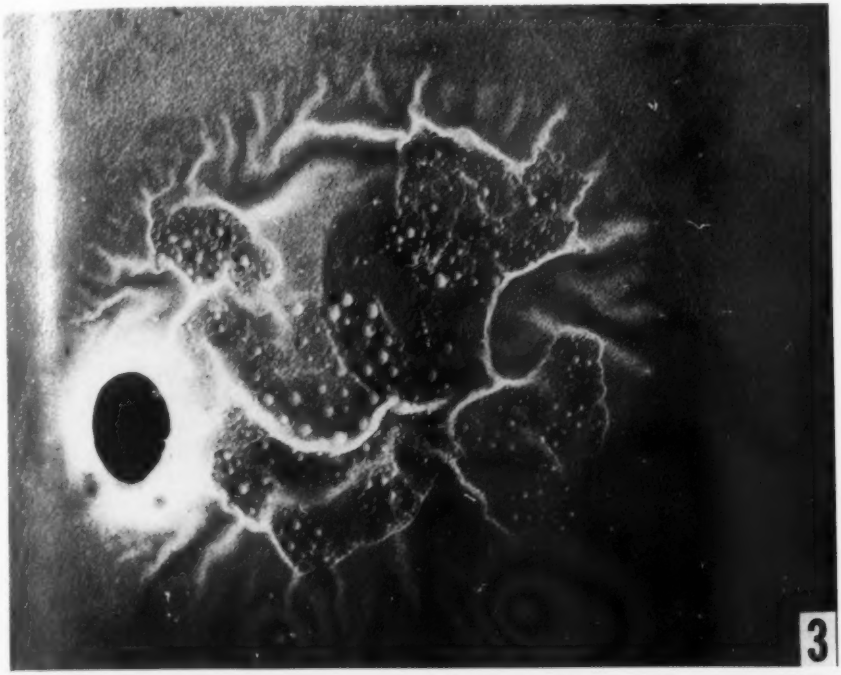
PLATE 23

FIGURE 3.—Cigarette smoke. Middle of cigarette. Diluted 200 times. × 25,000

FIGURE 4.—Cigar smoke. Half length. Diluted 15 times. × 25,000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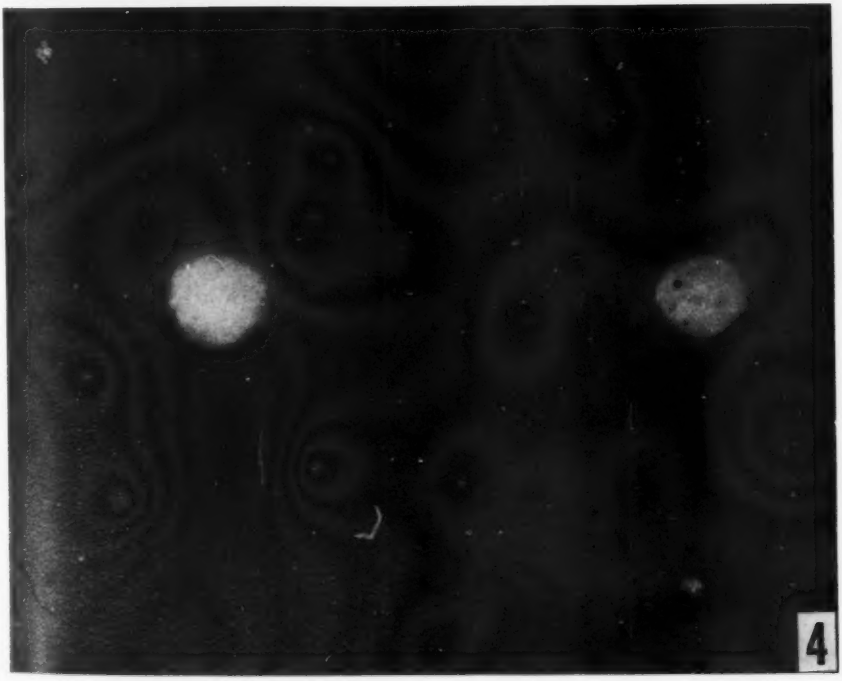
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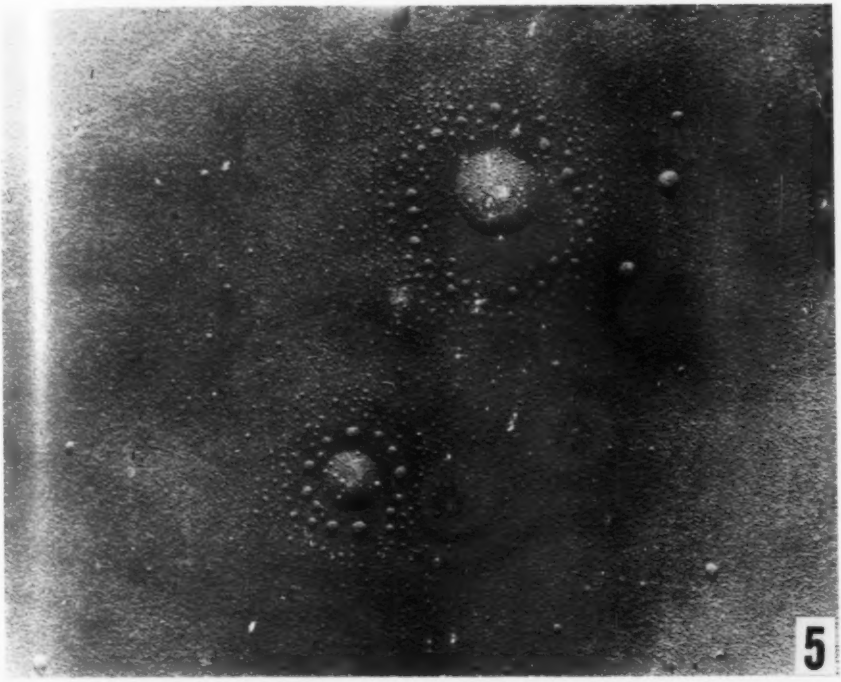
PLATE 24

FIGURE 5.—Cigar smoke. Half length. Diluted 200 times. × 25,000

FIGURE 6.—Cigar smoke. Third puff. Diluted 15 times. × 25,000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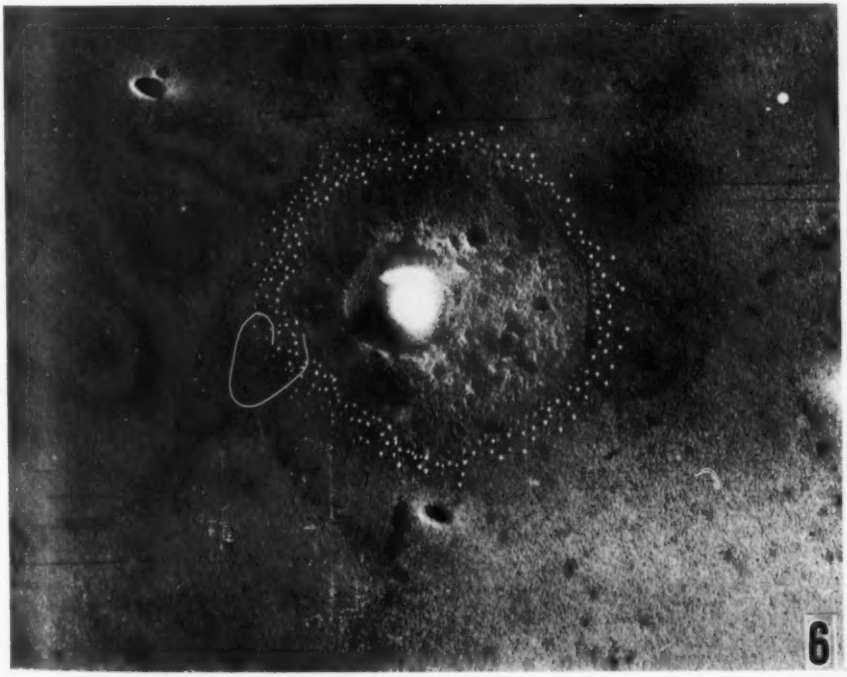
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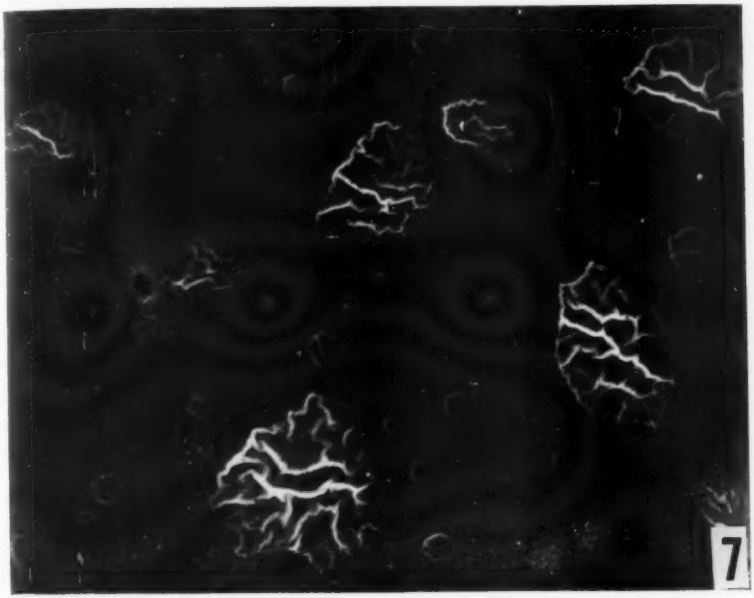
PLATE 25

FIGURE 7.—Screen inserted in cigarette. × 13,000

FIGURE 8.—Inhaled-exhaled cigarette smoke. × 53,000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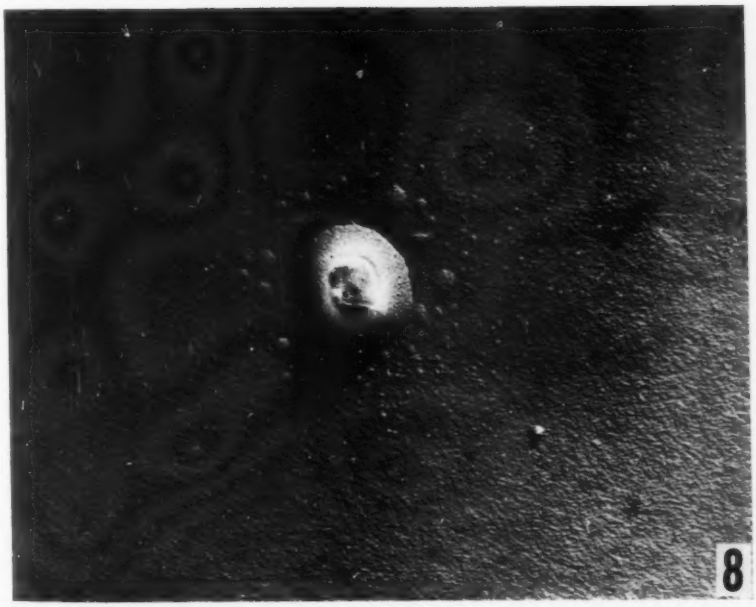
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Virus of Avian Erythroblastosis. III. Antigenic Constitution and Relation to the Agent of Avian Myeloblastosis [1,2]

DOROTHY BEARD, GEORGE S. BEAUDREAU, ROBERT A. BONAR, D. G. SHARP, *and* J. W. BEARD, *Department of Surgery, Duke University School of Medicine, Durham, North Carolina*

The application of standard serologic techniques has been very productive in the clarification of certain aspects of the biologic properties and behavior of the virus of one form of avian leukemia, myeloblastosis (*1*). Immune serums produced in chickens with the agent were effective in the neutralization of specific infectivity and precipitation of the particles identified as the virus. Analogous antibodies from heterologous hosts, principally the rabbit, bound complement with the virus and, likewise, were potent in the neutralization of infectious capacity. An unusual phenomenon was encountered in the observation that immunization of rabbits with normal chicken tissue (homogenates of liver and spleen) or serum protein resulted in antibodies which not only bound complement in the presence of the virus but were strongly active in the neutralization reaction. A surprising development was the discovery that Forssman antibody induced in the rabbit with homogenate of guinea-pig kidney likewise neutralized the virus, though to a lesser degree than that seen with the other immune serums. These results were interpreted to indicate the presence, as intrinsic virus components, of antigens not only peculiar to the agent (active in the homologous host) but also identical in behavior with normal chicken and Forssman antigens.

More recently, experiments have been undertaken to characterize the virus of another avian leukemia, erythroblastosis. The objectives of the work have been concerned not only with the properties of this agent *per se* but also with the search for criteria that permit interpretations relative to similarities and differences between the viruses of erythroblastosis and myeloblastosis. The results obtained earlier have been described in other reports (*2,3*). In this paper are presented the findings obtained in serologic and immunologic studies on the virus of erythroblastosis, carried out in both direct and cross reactions involving erythroblastosis and myeloblastosis viruses with their respective antiserums from chickens and rabbits. The experiments have shown that the agents of the 2 leukemias

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are closely related, though, on the basis of this and other work, distinctly different biologic entities.

Materials and Methods

The strain of erythroblastosis virus employed here was that designated "R" by Dr. Engelbreth-Holm (*4,5*) by whom it was derived originally, in 1930, from a Plymouth Rock hen showing myeloid leukosis. The characteristics of the original disease and the subsequent development of an essentially pure strain of erythroblastosis have been described at intervals (*4*) since then. The actual starting material for the experiments at Duke was sent, as already described (*3*), by Dr. Astrid Fagraeus. This strain was chosen because of the extensive experience of other workers (*4,6–8*) with the disease in European laboratories. The first passages and the characteristics of the disease in this country have already been indicated (*3*), and there has been no evident change on further passage with *filtered plasma*. There was no apparent difference between this disease and the strain described by Furth (*9*).

Experiments involving myeloblastosis virus were made with the same BAI strain A employed in all of the earlier work [*see* (*1*) and (*10*) for references]. It should be emphasized that the disease is a pure strain of myeloblastosis (*11*), designated erroneously in previous reports as erythromyeloblastosis because of the lack, at that time, of first-hand experience with erythroblastosis. Under the conditions of the experiments in this laboratory, this form of leukosis, myeloblastosis (*12,13*), differs radically in pathology, host response, and the attributes of the causative agents from the erythroblastosis under examination.

Virus.—Erythroblastosis virus employed in the neutralization experiments was that present in a pool of plasma from chickens with the disease. This was derived from 11 of 30 chickens inoculated at 40 days of age with 0.1 ml. of undiluted and filtered previous-passage plasma. Birds of this age and older are more susceptible to erythroblastosis virus (*3,14*) than younger ones, contrary to the finding with myeloblastosis (*15*). Blood was taken by heart puncture (*16,17*) from 4, 3, and 4 birds on the 8th, 9th, and 10th days, respectively, after inoculation, shortly before death of each chicken was expected. Cells were removed by centrifugation of the heparinized blood—each specimen separately—first at 1000 × g for 10 minutes and then at 1500 × g for 15 minutes. The individual plasmas were frozen separately in a dry ice-alcohol bath and stored in crushed dry ice until all had been collected. On November 14, 1955, the specimens were thawed, pooled, and centrifuged again for 20 minutes at 1500 × g. The well-mixed pool, designated as "J," was then parceled in 5.0 and 2.0 ml. volumes in small-mouth rubber-stoppered serum vials, refrozen quickly, and stored again covered with crushed or pulverized dry ice (−78° C.). At the time of use, a specimen was thawed, centrifuged again, and then filtered with or without dilution through a celite mat, followed by passage through a small Selas candle of 02 porosity (*16,17*). During the interval to July 2, 1956, there was no definite evidence of systematic

decline in infectious capacity, although shifts in end point were observed that may have been due to host variation, as described elsewhere (*18*).

Myeloblastosis virus was prepared [3] also in a pool (Pool C) in a like manner from plasmas derived in the usual way (*6,7*) after selection of donors of plasma high in virus content by means of the micro adenosinetriphosphatase test (*19*). The plasmas were pooled after centrifugation separately to remove the cells, and aliquots of 2.0 or 5.0 ml. were kept in small vials in dry ice as described above. After a specimen had been thawed, the fluid was centrifuged again and filtered through celite and Selas candles of 02 porosity.

Titration of virus infectivity.—Titration of control and serum-virus mixtures was effected by the latent-period procedures applicable, respectively, to the erythroblastosis (*3*) and myeloblastosis (*20*) viruses. The hosts were always White Leghorn birds of line 15 developed (*21,22*) at the Regional Poultry Research Laboratory, East Lansing, Michigan. Some were hatched from eggs shipped by air from East Lansing, but most came from a laying flock at the laboratory. The volume of the inoculums was 0.1 ml., and the injections were made into the wing vein. Chicks 3 to 5 days of age (*15,20*) were used for the myeloblastosis virus. In the instance of erythroblastosis, the age at inoculation was 16 to 18 days (*3,14*) after hatching. All birds in a given experiment were of the same hatch.

Immune serums.—All the immune serums, except 2, were aliquots of those employed in the previously reported experiments on the myeloblastosis virus; their preparation has already been described (*1*). They had been kept at −22° C. One of the new serums was prepared in chickens with erythroblastosis virus and the other was an anti-Forssman immune serum from the rabbit.

Antierythroblastosis-virus immune serum from chickens was prepared (*23*) with the use of the formalin-inactivated agent in concentrates obtained by centrifugation. Because of the relatively low concentration of virus in plasma—few contained more than about 10^{10} particles per ml. in the best specimens—the need for agent from considerable volumes of plasma was anticipated. Accordingly, chickens 2 to 20 weeks of age were inoculated with virus and bled from the heart in the usual way shortly before death was expected. The cells were removed by repeated low-speed centrifugation, and the resulting plasma was then spun at 20,000 × g for 60 minutes. The sedimented virus was resuspended in Ca-free Ringer's solution at about 25 times concentration with respect to the initial volume of plasma. Formalin in Ca-free Ringer's solution was added in amounts to bring the suspension to 10 to 20 times concentration relative to virus and 0.1 percent formalin. The suspensions were stored in the refrigerator for about 3 weeks. This vaccine was prepared in

[3] In all of the earlier work, 3-day-old chicks were used for all titrations of myeloblastosis virus, and specimens of virus were obtained from the birds that became infected. Recent experience has shown that blood from chicks inoculated at 10 days of age contains as great a concentration of virus as that of the younger animals and, in addition, the volumes of plasma are much greater. Although fewer of the 10-day-old chicks become diseased, the collection of virus (not titration) is greatly facilitated by use of the older birds.

batches, at intervals, from a total of 2125 ml. of plasma from 198 chickens, and all of it was used for the injection of 8 White Leghorn (line 15) roosters of 6.5 months of age. All injections were made into the peritoneal cavity. The first course consisted of two 1.0 ml. injections on alternate days. A week later 3 injections of 2.0 ml. doses were made on alternate days. The final course, after skipping 1 week, was 2.0, 2.5, and 2.5 ml. given in the same way. All the birds, none of which showed evidence of disease, were bled from the heart 11 days after the last injection. The serums were recovered, pooled in a single lot, parceled in small aliquots, and stored at −22° C.

Forssman antibody was produced in rabbits with guinea-pig kidney ground with sand in a mortar. A 10 percent suspension of Ca-free Ringer's solution, freed of sand and large tissue masses by low-speed centrifugation, was divided into small lots and stored at −22° C. The preparation, thawed as needed, was given in 2 c.c. volumes intraperitoneally for 3 courses of 3 injections on alternate days with 1 week between courses. The rabbits were bled 11 days after the last injection. The serums were obtained, pooled, and stored in 2.0 and 5.0 ml. volumes at −22° C.

Neutralization.—In each experiment, 2 to 4 dilutions of the filtered virus preparations were prepared as indicated in the respective tables. Dilution for filtration, 1 : 1 or 1 : 2, was made with Ca-free Ringer's solution, and all subsequent dilutions were made with BSA-Simms' solution (*24*). The diluent, Simms' solution containing 1.5 percent bovine serum albumin, was prepared from time to time and kept frozen at −22° C. Appropriate volumes were thawed just before use. The serum-virus mixtures consisted of 1.0 ml. of the appropriate dilution of serum, 1.0 ml. of the desired dilution of virus, and 3.0 ml. of BSA-Simms' solution. In the tables and text-figures, *virus* and *serum amount* or *volume* refer to volume per inoculum (0.1 ml.). For example, *virus* 10^{-2} in text-figure 1 means that where the mixtures contained a plasma concentration of 10^{-2}, in the inoculum of 0.1 ml. there was 10^{-3} ml. of plasma. When undiluted serum was used, the volume of serum per 0.1 ml. inoculum was 20λ. Normal serum was used as control in some of the experiments, but in most instances the base line of infectious capacity was established by the responses to the various dilutions of virus without normal serum. In those instances in which serum control was used, as can be seen in the appropriate tables, the responses to the normal-serum mixtures were averaged with those to the virus dilutions containing none to yield the base.

The mixtures, 5.0 ml. total volume, were made in Wassermann tubes, as follows: The diluent was added first, followed by immune serum, and finally, after the 2 were stirred without wetting the upper part of the tube, the virus was added. Mixing was effected by inversion of the tubes, closed with parafilm, several times, after which the tubes were closed with tightly fitting rubber stoppers. The tubes were immersed well above the fluid level in a water bath at 37° C. for 2 hours. All control tubes received the same treatment, and the fluids were not allowed to touch

the stoppers. Immediately after removal from the bath, the tubes were chilled and kept immersed in cracked ice until the mixtures could be injected.

All serums were heated at 56° C. for 30 minutes before use in the mixtures. Except in the Forssman studies, the rabbit serums were always absorbed 4 times with packed, washed sheep erythrocytes as previously described (*1*). In the experiment with Forssman antibody, only the absorbed serum received this treatment.

Precipitation.—The preparations of erythroblastosis virus employed for the precipitin studies were concentrates obtained by centrifugation of plasma, as already described (*23*). Myeloblastosis virus was that in plasma selected on the basis of high enzymatic activity to dephosphorylate adenosinetriphosphate. Experiments were made with serum from normal chickens, with the antimyeloblastosis and antierythroblastosis chicken serums. All the serums were heated at 56° C. for 30 minutes and were cleared of particulate material by centrifugation at 47,000 × g for 30 minutes. Only the upper two thirds of the supernatant serum was used. Mixtures were made of 1 part serum, 1 part virus plasma or concentrate, and 1 part NaCl solution to give the final concentration of 7.5 percent NaCl (*25*). The mixtures were brought to 37° C. for 2 hours, sampled at intervals, kept at 2° C. overnight, and then sampling was continued. The samples were diluted with 0.9, 4, or 8 percent NaCl solution, and the particles or agglomerates were sedimented onto an agar surface for examination, as described earlier (*1*).

Enumeration of virus particles.—Counts were made of the erythroblastosis-virus particles in Pool J on 4 different occasions. The values were 6.0, 6.6, 9.0, and 7.4×10^{10} with an average of 7.2×10^{10} particles per ml. of whole plasma. The count on the pool, C, of myeloblastosis virus was 9.5×10^{11} particles per ml. Such examinations of preparations of erythroblastosis virus usually did not appear to yield results as accurate as those obtained with myeloblastosis because of the relatively large amount of amorphous material in preparations of relatively low virus-particle content. The technique employed was that involving sedimentation of the particles on agar (*26*), fixation with osmic acid, and preparation of pseudoreplicas with collodion from which counts were made, as in the case of myeloblastosis (*16*).

Experiments and Results

The experiments may be grouped for convenience of presentation and differentiation into 4 categories: 1) neutralization of the virus of erythroblastosis with homologous chicken immune serum; 2) neutralization or treatment of the virus of erythroblastosis with serum from the rabbit immunized with normal chicken tissue or protein and with Forssman antigen; 3) cross-neutralization of the viruses of erythroblastosis and myeloblastosis with antiviral immune serums from the chicken and rabbit; and 4) direct- and cross-precipitin reactions with the viruses and their respective immune serums from the *chicken*.

One of the principal problems of the work, as might be expected, was to establish the proper proportions of immune serum and virus for appropriate demonstration of the respective reactions. In the earlier work with myeloblastosis (*1*), in which a different preparation of virus was used for each study, it was often necessary to do checkerboard experiments employing several dilutions of virus, assayed by ATPase activity, in order to include the appropriate relations. This could be avoided, for the most part in the present investigation, by using pooled virus preserved by freezing at −78° C. Although it was not known in the beginning that this was feasible, it was learned (*18*) that the erythroblastosis and myeloblastosis viruses, like the Rous sarcoma agent (*27*), were either quite stable or, at least, changed but little under these conditions. Thus, the infectious activity could be determined in advance and the experiments easily planned. All the neutralization experiments involving the erythroblastosis virus, reported in this paper, were made with Pool J. The pattern of host response to this preparation, which contained about 7.2×10^{10} virus particles per ml., was identical with that to the virus in experiment 165 [table 1 and text-fig. 3 of reference (*3*)] with the exception that, on the average in the experiments described here, the infectious capacity of Pool J was approximately 10-fold greater (on the basis of latent-period relations) than that of the virus preparation of experiment 165. The infectious activity of the pool of myeloblastosis virus, Pool C, containing 9.5×10^{11} virus particles per ml., was very close to that of the virus previously used in the experiment shown in table 10 and text-figure 14 of (*1*), in which the virus-particle concentration was 10^{12} per ml.

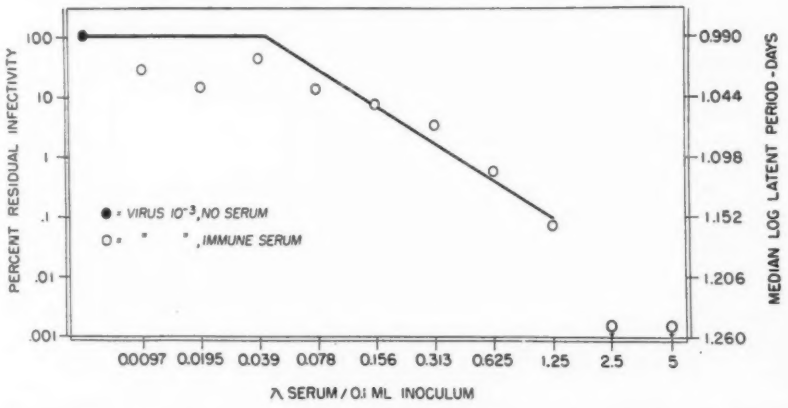
1. Neutralization of erythroblastosis virus with homologous immune serum from the chicken.—This experiment was made with the immune serum obtained from chickens given repeated injections of the formalin-inactivated erythroblastosis-virus concentrate. The plan of the experiment and the observed data are given in table 1, and the results are summarized graphically in text-figure 1. The control preparation in this study consisted of virus alone, and no normal-serum controls were included. It is seen that the infectious activity of the preparation was such that 0.005 λ of virus was sufficient to kill essentially all the individuals of the dose group. The serum was of such potency that 2.5 λ could neutralize, almost completely, the 200 lethal doses, and the total reduction in infectivity, based on measurable end point, was at least 100,000-fold. The results of the experiment demonstrate the efficiency of the formalin-inactivated virus, when given in proper amounts, to induce potent neutralizing antibodies in the homologous host.

2. Neutralization of the virus of erythroblastosis with serum from rabbits immunized with normal chicken material and treatment of the virus with Forssman antibodies from the rabbit.—(*a*) The virus of myeloblastosis is strongly neutralized by the serum of rabbits immunized with homogenates of normal chicken tissue [table 6 and text-fig. 10 of (*1*)]. A test was made to learn if the same immune serum employed in the experiments with myeloblastosis virus would likewise neutralize the virus of erythroblastosis.

TABLE 1.—*Neutralization of virus of erythroblastosis in filtered plasma (Pool J) with serum from chickens immunized with formalin-inactivated concentrate of virus of erythroblastosis* (see *text-fig. 1*)

Serum	Volume of plasma containing virus* (λ)	Volume of serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity‡ (*percent*)
None	5	—	30	30	0. 958	—
	0. 5	—	30	29	1. 035	—
	0. 05	—	30	30	1. 035	—
	0. 005	—	30	29	1. 099	—
Antiviral	1	20	30	3	>1. 3	<0. 0002
	"	10	30	1	>1. 3	<0. 0002
	"	5	30	4	>1. 3	<0. 0002
	"	2. 5	30	9	>1. 3	<0. 0002
	"	1. 25	30	22	1. 160	0. 09
	"	0. 625	30	24	1. 111	0. 57
	"	0. 313	30	28	1. 069	3. 4
	"	0. 156	30	28	1. 050	7. 8
	"	0. 078	30	30	1. 036	14
	"	0. 039	29	28	1. 010	43
	"	0. 0195	30	28	1. 036	14
	"	0. 0097	30	28	1. 020	28

*Per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*5*) for method of calculation].
‡Calculated on the basis of latent-period relations.



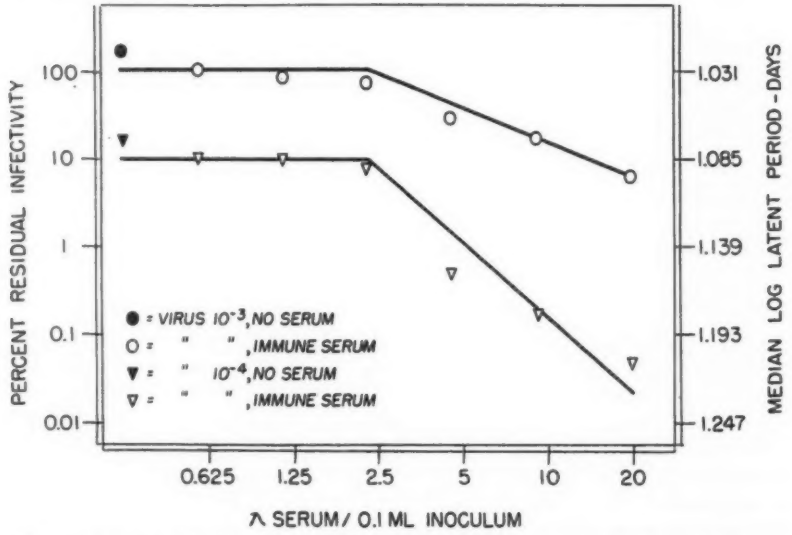
TEXT-FIGURE 1.—Neutralization of the virus of erythroblastosis in filtered plasma (Pool J) with serum from chickens immunized with formalin-inactivated concentrate of the virus of erythroblastosis (*see* table 1).

The results of such a study (table 2) are shown graphically in text-figure 2. In this case, 2 dilutions of virus were employed, with duplicate dilutions of immune serum. There were also 2 dilutions of normal rabbit serum with each quantity of virus. The base line of 100 percent activity was established with the median log latent-period values obtained with both untreated virus and the mixtures containing normal serum. The latent-

TABLE 2.—*Neutralization of virus of erythroblastosis in filtered plasma (Pool J) with serum from rabbits immunized with homogenate of normal chick tissue* (see *text-fig. 2*)

Serum	Volume of plasma containing virus* (λ)	Volume of serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity‡ (*percent*)
None	5	—	30	27	0. 975	—
	0. 5	—	28	28	1. 042	—
	0. 05	—	29	28	1. 090	—
Anti-chick-tissue	1	20	28	27	1. 105	6. 5
	"	8. 8	29	28	1. 074	16
	"	4. 4	29	28	1. 060	29
	"	2. 2	30	30	1. 037	77
	"	1. 1	23	23	1. 034	87
	"	0. 55	28	28	1. 030	104
Normal	1	20	28	28	1. 027	159
	"	8. 8	29	28	1. 041	65
Anti-chick-tissue	0. 1	20	29	21	1. 211	0. 05
	"	8. 8	28	22	1. 181	0. 18
	"	4. 4	25	24	1. 177	0. 55
	"	2. 2	28	28	1. 092	8. 1
	"	1. 1	30	30	1. 085	10. 8
	"	0. 55	30	29	1. 085	10. 8
Normal	0. 1	20	29	29	1. 073	18
	"	8. 8	26	21	1. 115	3

*Per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*3*) for method of calculations].
‡Calculated on the basis of latent-period relations.



TEXT-FIGURE 2.—Neutralization of the virus of erythroblastosis in filtered plasma (Pool J) with the serum from rabbits immunized with homogenate of normal chick tissue (*see* table 2).

period values observed with normal serum are given in table 2 but were not plotted in text-figure 2.

The results show that the rabbit antinormal-chick-tissue immune serum was effective in the neutralization of both quantities of virus, but much more so, as would be expected, for the higher dilution.

(*b*) Immune serums produced in the rabbit by injections with normal chicken serum were also active in neutralizing the virus of myeloblastosis [table 7 and text-fig. 10 of (*1*)]. Such was the case, too, with the virus of erythroblastosis, as indicated in table 3 and text-figure 3. In this experiment the degree and pattern of neutralization of the erythroblastosis virus were essentially identical with those observed in the preceding experiment with antinormal-chicken-tissue rabbit immune serum.

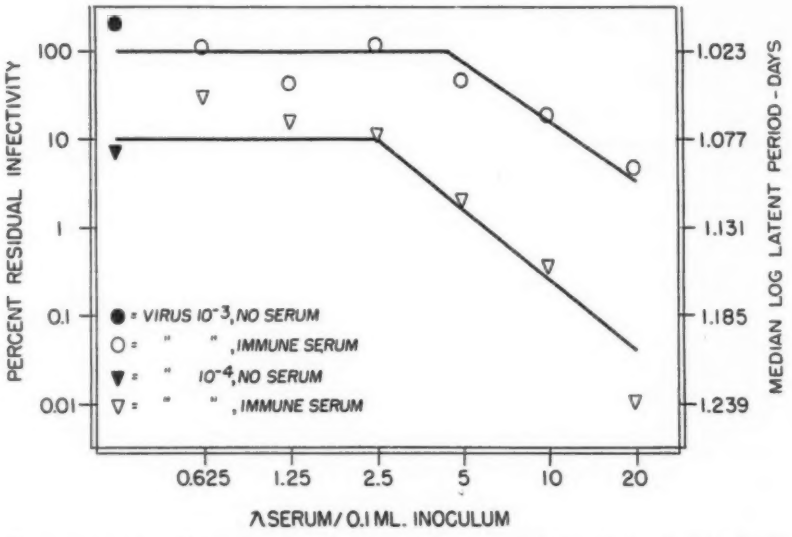
It should be emphasized that the rabbit immune serums employed here were the same as those used in the study of myeloblastosis. It would appear that both serums, the one against normal chicken tissue and the other against normal chicken serum, were almost equally potent against the erythroblastosis virus (*cf.* text-fig. 2 with text-fig. 3) and, in addition, the percentage of neutralization was about the same with erythroblastosis as with myeloblastosis [text-fig. 9 of (*1*)]. However, it should be remembered that the actual number of virus particles of erythro-

TABLE 3.—*Neutralization of virus of erythroblastosis in filtered plasma (Pool J) with serum from rabbits immunized with serum from normal chickens* (see *text-fig. 3*)

Serum	Volume of plasma containing virus* (λ)	Volume of serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity‡ (*percent*)
None	5	—	30	29	0. 992	—
	0. 5	—	30	29	1. 025	—
	0. 05	—	30	27	1. 070	—
	0. 005	—	30	26	1. 115	—
Anti-chick serum	1	20	30	27	1. 095	5
	"	10	30	29	1. 062	19
	"	5	30	29	1. 040	48
	"	2. 5	30	30	1. 020	114
	"	1. 25	29	28	1. 042	45
	"	0. 625	29	29	1. 022	105
Normal	1	20	29	28	1. 022	105
	"	10	30	29	1. 042	45
Anti-chick serum	0. 1	20	30	14	1. 238	0. 0104
	"	10	28	23	1. 155	0. 57
	"	5	30	27	1. 115	2. 0
	"	2. 5	30	30	1. 075	11
	"	1. 25	29	29	1. 065	17
	"	0. 625	30	29	1. 050	32
Normal	0. 1	20	30	29	1. 085	7
	"	10	30	28	1. 085	

*Per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*3*) for method of calculation].
‡Calculated on the basis of latent-period relations.

blastosis was of the order of 100 to 1000 times less than the number present in the myeloblastosis plasma [*see* tables 6 and 7 of (*1*)]. Thus, it really is not possible to compare directly the results with the 2 viruses on a quantitative basis, but the conclusion can be drawn that both agents were effectively neutralized by the 2 serums.



TEXT-FIGURE 3.—Neutralization of the virus of erythroblastosis in filtered plasma (Pool J) with serum from rabbits immunized with serum from normal chickens (*see* table 3).

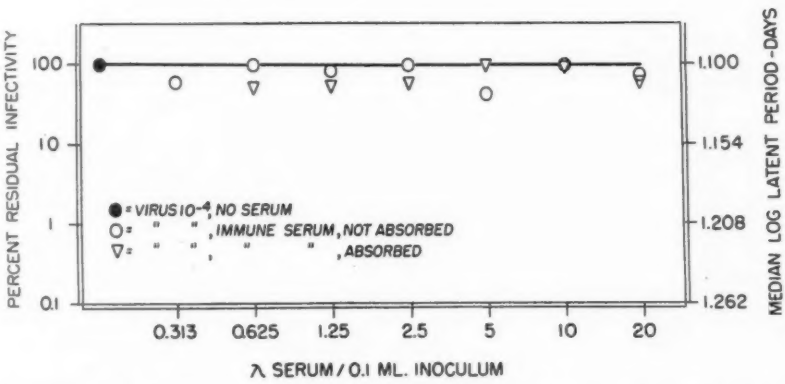
(*c*) A definite effect to neutralize the virus of myeloblastosis was exerted (*1*) by anti-Forssman immune bodies from rabbits given homogenate of guinea-pig kidney. This influence, which is present in serum heated at 56° C. for 30 minutes, can be removed completely by repeated absorption of the serum with sheep red blood cells [table 9 and text-fig. 13 of (*1*)]. Whereas the viruses of myeloblastosis and erythroblastosis did not differ qualitatively in their response to antinormal-chicken-tissue and protein immune serums from the rabbit, an *outstanding difference* was shown in their behavior in the presence of Forssman antibodies. This is illustrated in the results of the experiment of table 4 and text-figure 4, in which it is seen that *no neutralization* of the virus of erythroblastosis was effected by the same Forssman antibody employed in the study of myeloblastosis. It should be noted that in this experiment the virus per inoculum was 10^{-4} ml., which was the highest dilution compatible with dose-response analysis. Identical results were observed with the new anti-Forssman immune serum. In this respect then, the viruses reveal a remarkable apparent difference in serologic behavior.

3. Cross-neutralization experiments.—For these studies there were available an antimyeloblastosis chicken immune serum (2/23) and an antimyelo-

TABLE 4.—*Treatment of virus of erythroblastosis in filtered plasma (Pool J) with serum from rabbits (AB) [the same as that employed with myeloblastosis virus, table 9 and text-fig. 13 of reference (1)] immunized with homogenate of guinea-pig kidney (Forssman antigen) and with same serum after absorption with sheep red blood cells* (see *text-fig. 4*)

Serum	Volume of plasma containing virus* (λ)	Volume of serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity‡ (*percent*)
None	5. 0	—	30	30	1. 015	—
	0. 5	—	30	30	1. 082	—
	0. 05	—	29	27	1. 110	—
	0. 005	—	30	25	1. 147	—
Anti-Forssman (not absorbed)	0. 1	20	29	28	1. 092	71
	"	10	30	30	1. 099	96
	"	5	30	30	1. 120	43
	"	2. 5	30	30	1. 101	96
	"	1. 25	30	30	1. 095	81
	"	0. 625	30	27	1. 101	96
	"	0. 313	30	27	1. 111	63
Anti-Forssman (absorbed with sheep red blood cells)	0. 1	20	30	29	1. 090	65
	"	10	29	29	1. 101	96
	"	5	30	30	1. 101	96
	"	2. 5	30	29	1. 111	63
	"	1. 25	30	29	1. 115	53
	"	0. 625	30	29	1. 115	53

*Per 0.1 ml. inoculum.
†Correct number of chicks inoculated [*see* (*3*) for method of calculation].
‡Calculated on the basis of latent-period relations.



TEXT-FIGURE 4.—Treatment of virus of erythroblastosis in filtered plasma (Pool J) with the serum from rabbits (AB) [the same as that employed with myeloblastosis virus, table 9 and text-fig. 13 of reference (*1*)] immunized with homogenate of guinea-pig kidney (Forssman antigen) and with the same serum after absorption with sheep red blood cells (*see* table 4).

blastosis rabbit immune serum, both of which were the same as those used before (*1*). The antierythroblastosis chicken immune serum prepared in the present work was also employed.

(*a*) The influence on the virus of erythroblastosis of antimyeloblastosis chicken immune serum is seen in table 5 and text-figure 5. In this experiment a relatively low dilution ($10^{-2.176}$ ml. of plasma in the chicken) of virus plasma was employed in the test mixtures (6.66λ per 0.1 ml. inoculum). The amount of virus was thus 6.66-fold greater than that employed for neutralization by the homologous antiserum (table 1, text-fig. 1). Nevertheless, it is evident that the serum seemed fully as active in neutralizing the erythroblastosis virus (discounting the number of virus particles involved) as the antierythroblastosis chicken immune serum, if not more so. This experiment shows quite definitely that the 2 agents are closely related serologically. Normal chicken-serum controls were included in this experiment, and the results obtained are seen in table 5.

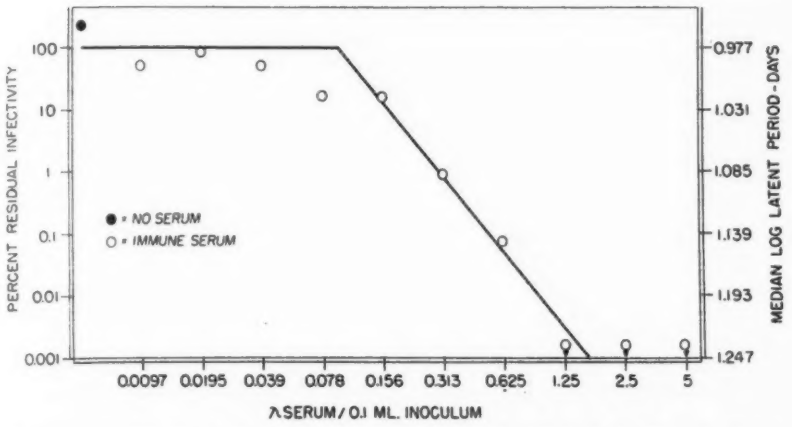
(*b*) A result similar in kind was obtained when the virus of erythroblastosis was exposed to antimyeloblastosis-virus immune serum from the rabbit as seen in table 6 and text-figure 6. It will be recalled that this immune serum was prepared (*1*) by repeated injections of rabbits with concentrates of the virus of myeloblastosis and that the serum had been absorbed repeatedly with sheep red blood cells. The serum obviously was highly potent in the neutralization of the heterologous agent.

(*c*) The converse reaction, that is, that between the virus of myeloblastosis and antierythroblastosis immune serum from chickens seemed much less impressive (table 7 and text-fig. 7). Nevertheless, the effect of

TABLE 5.—*Neutralization of virus of erythroblastosis in filtered plasma (Pool J) with serum (2/23) [this was the same serum as that employed with myeloblastosis virus, table 10 and text-fig. 14 of reference* (1)*] from chickens hyperimmunized with virus of myeloblastosis* (see *text-fig. 5*)

Serum	Volume of plasma containing virus* (λ)	Volume of serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity‡ (*percent*)
None	6.66	—	29	25	0.958	232
	0.66	—	30	30	1.03	—
	0.066	—	30	29	1.058	—
Antiviral	6.66	5.0	30	2	—	<0.001
	"	2.5	30	2	—	<0.001
	"	1.25	29	13	1.285	<0.001
	"	0.625	30	27	1.145	0.077
	"	0.313	29	28	1.082	1.1
	"	0.156	29	29	1.020	16
	"	0.078	30	30	1.018	17
	"	0.039	30	30	0.992	53
	"	0.0195	30	30	0.981	88
	"	0.0097	29	29	0.992	53
Normal	6.66	5.0	29	27	0.980	88
	"	2.5	30	29	0.992	53

*Per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*3*) for method of calculation].
‡Calculated on the basis of latent-period relations.



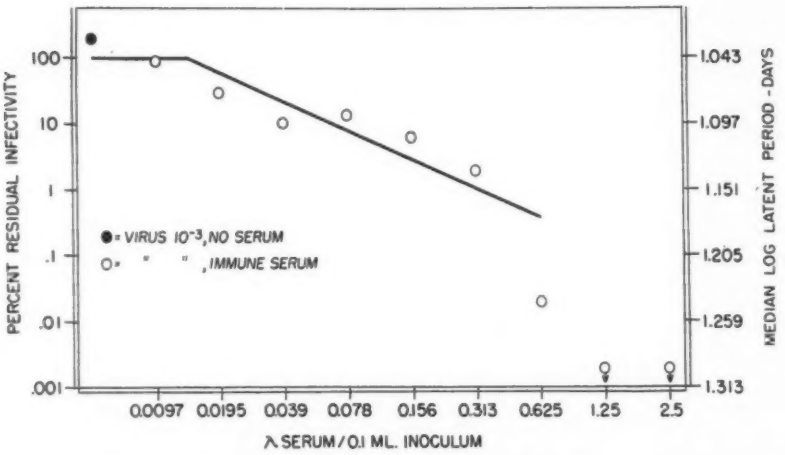
TEXT-FIGURE 5.—Neutralization of the virus of erythroblastosis in filtered plasma (Pool J) with serum (2/23) [this was the same serum as that employed with myeloblastosis virus, table 10 and text-fig. 14 of reference (*1*)] from chickens hyperimmunized with the virus of myeloblastosis (*see* table 5).

TABLE 6.—*Neutralization of virus of erythroblastosis in filtered plasma (Pool J) with serum of rabbits [the same as that employed with myeloblastosis virus, table 5 and text-fig. 8 of reference* (1)] *immunized with virus of myeloblastosis* (see *text-fig. 6*)

Serum	Volume of plasma containing virus* (λ)	Volume of serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity‡ (*percent*)
None	5	—	30	30	0. 995	—
	0. 5	—	30	29	1. 040	—
	0. 05	—	30	28	1. 095	—
	0. 005	—	30	28	1. 135	—
Antiviral	1	10	29	0	>1. 3	<0. 0002
	"	5	29	2	>1. 3	<0. 0002
	"	2. 5	28	1	>1. 3	<0. 0002
	"	1. 25	30	1	>1. 3	<0. 0002
	"	0. 625	29	13	1. 245	0. 018
	"	0. 313	28	27	1. 136	1. 9
	"	0. 156	28	27	1. 109	6
	"	0. 078	28	27	1. 091	13
	"	0. 039	28	27	1. 097	10
	"	0. 0195	29	28	1. 072	29
	"	0. 0097	26	25	1. 045	92
Normal	1	10	27	27	1. 045	92
	"	5	30	28	1. 057	55

*Per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*3*) for method of calculation].
‡Calculated on the basis of latent-period relations.

the serum, for example, in the mixture containing 0.74λ virus per inoculum, was such that about 99 percent of virus infectivity was neutralized. This effect of the erythroblastosis immune serum on the virus of myeloblastosis was obviously less, about 10-fold, than that observed earlier in tests with



TEXT-FIGURE 6.—Neutralization of virus of erythroblastosis in filtered plasma (Pool J) with serum of rabbits [the same as that employed with myeloblastosis virus, table 5 and text-fig. 8 of reference (*1*)] immunized with the virus of myeloblastosis (*see* table 6).

antimyeloblastosis immune serum against the myeloblastosis virus [table 10 and text-fig. 14 of (*1*)].

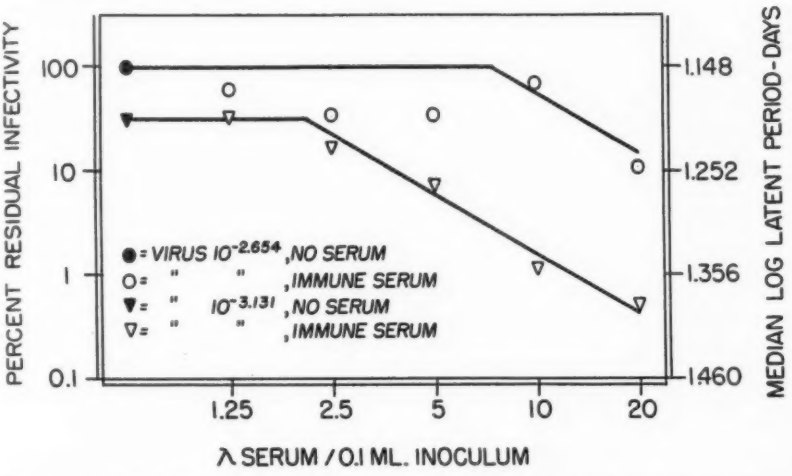
4. Precipitin reactions.—The results of the foregoing experiments showed clearly that the viruses of erythroblastosis and myeloblastosis are antigenically related with respect to the induction of and reaction with specific antiviral neutralizing antibodies in the chicken and in cross reaction in one direction with antiviral immune serum from the rabbit. It was seen, also, that they were related in antigenic structure in their qualitatively similar reactions with other serums produced in the rabbit with normal host material. There was a definite *difference* only in the lack of neutralizing effect on the virus of erythroblastosis by Forssman antibody.

In these experiments there was no ready means for quantitating any possible difference in specific viral constitution. Some opportunity was afforded for that, however, on the basis of direct- and cross-precipitin reactions with the respective chicken immune serums. It had been observed (*1*) that with the virus of myeloblastosis strong precipitin reactions could be obtained with homologous chicken immune serum. Accordingly, it was thought probable that a like reaction might occur between the virus of erythroblastosis and its immune serum, if one of sufficient potency could be produced. That such was the case was demonstrated, as already briefly reported (*23*), by use of the immune serum induced in the chicken with formalin-inactivated erythroblastosis virus and employed in the neutralization experiments described above. These studies on precipitation of the erythroblastosis virus served 2 principal purposes, in showing first, that precipitating antibody *can* be produced by the amounts of virus available; and, second, that the reaction could be employed as a specific criterion in identifying the particles observed in

TABLE 7.—*Neutralization of virus of myeloblastosis in filtered plasma with serum from chickens immunized with formalin-inactivated concentrate of virus of erythroblastosis* (see *text-fig. 7*)

Serum	Volume of plasma containing virus* (λ)	Volume of serum* (λ)	Chicks inoculated†	Chicks positive	Median log latent period (*days*)	Residual infectivity‡ (*percent*)
None	11. 1	—	27	26	1. 062	—
	1. 11	—	27	20	1. 145	—
Antiviral	2. 22	20	29	18	1. 247	11
	"	10	29	24	1. 165	69
	"	5	25	22	1. 196	34
	"	2. 5	28	24	1. 196	34
	"	1. 25	29	22	1. 172	59
Antiviral	0. 74	20	30	11	1. 382	0. 18
	"	10	30	15	1. 351	1. 1
	"	5	30	14	1. 272	6. 4
	"	2. 5	28	21	1. 228	17
	"	1. 25	25	18	1. 198	33

*Per 0.1 ml. inoculum.
†Corrected number of chicks inoculated [*see* (*1*) for method of calculation].
‡Calculated on the basis of latent-period relations.



TEXT-FIGURE 7.—Neutralization of virus of myeloblastosis in filtered plasma with serum from chickens immunized with formalin-inactivated concentrate of the virus of erythroblastosis (*see* table 7).

plasma as the virus itself. This was of special importance because many of the physical and chemical procedures available for identification of the virus of myeloblastosis were lacking or cumbersome to apply to the small amounts of erythroblastosis virus available.

The outcome of a precipitin reaction between erythroblastosis virus and its immune serum from the chicken is illustrated in figures 1 and 2.

An untreated concentrate of the virus is shown in figure 1. The appearance of the aggregates forming on treatment with the immune serum, in the presence of NaCl at 7.5 percent concentration, is shown in figure 2. It was notable that precipitation was only partial and could scarcely be detected by macroscopic examination. Yet reactions of this sort were obtained upon repetition of experiments.

In marked contrast to this result was the highly potent precipitative action of antimyeloblastosis chicken immune serum on the myeloblastosis virus (*1*). The virus present in plasma, as shown in figure 3, was completely precipitated under the same conditions (and in the same experiment) as those yielding only partial precipitation of the virus of erythroblastosis. This reaction between myeloblastosis virus and its antiserum was very rapid, whereas that with erythroblastosis was very slow. Such reactions between the 2 agents and their antiserums might be thought to be related to differences in the potency of the respective immune serums or perhaps to depend on quantitative differences in the constituents of the virus preparations. That the difference was probably due, instead, to differences in antigenic constitution is clearly suggested in figures 5 and 6. In figure 5 is shown the effect of the antimyeloblastosis-virus chicken immune serum (used in the mixture that resulted in the material shown in fig. 4) on the same concentrate of erythroblastosis virus that gave the result in figure 2. It is seen that the myeloblastosis immune serum (fig. 4) was active only in partial precipitation of the virus of erythroblastosis. The difference in antigenicity is shown still further in figure 6, which is an electron micrograph of the preparation of myeloblastosis virus (fig. 3) treated with antierythroblastosis immune serum. This is the same virus used with the myeloblastosis immune serum (fig. 4). In figure 6 there is barely a suggestion of a precipitin reaction, evident in the slightly greater particle aggregation in this preparation than in the plasma in figure 3.

Discussion

It was the purpose of the present work to establish a basis for the immunologic and serologic characterization of the virus of erythroblastic leukemia of chickens. In the process of the investigation, it was necessary first to provide the essential reactants, namely, antigen (the virus) and suitable immune serums. This was accomplished satisfactorily by recognition, based on earlier experience with the virus of myeloblastosis (*1*), of the problem of antigenic mass and by utilization of sufficient quantities of the virus source (plasma) for the purpose, as calculated by physical determination (particle count) of the virus content of plasmas from diseased birds. Administration of the required amount of virus inactivated with formalin resulted in the induction, in the small number of chickens used, of neutralizing and precipitating antibodies adequate for the objective of the investigations.

The findings, by methods which were simple and standard for dealing with similar problems with other viruses, serve to clarify much of the

earlier confusion in the field of immunologic experiments with various forms of the leukosis complex. The limited progress in such studies of the diseases, as in the instance of other chicken neoplasms, has been due in large part to lack of application, in the absence of knowledge about the nature and amount of virus, of the principles of antigenic or viral mass to the practical problems of immunization and serologic reactions. Much advance has been made (*28,29*) in the elucidation of other obscure problems related to viral mass and activity in the quantitative biologic experiments concerned with bioassay by Bryan and his co-workers with the Rous sarcoma virus. Some of the confusing results related to rabbit papillomatosis have likewise been discussed (*30*). It is entirely likely that the application of similar principles would afford rapid and definite progress in the study of other tumor viruses. The results of the various experiments emphatically demonstrate qualitative antigenic capacity of the avian-leukemia viruses in the induction of specific antibodies in the homologous host entirely in line with the well-known activity of the "ordinary" or non-tumor agents. In this respect again, as already established for chicken sarcoma (*31*) and rabbit papilloma viruses (*32*), there is much promise that other viruses causing tumors may be of like character.

An obvious question in the present work is that concerned with other aspects of antigenic constitution analogous to those observed with the virus of myeloblastosis. In that disease the virus was neutralized with immune serums from heterologous hosts, principally the rabbit, induced by injection of the animals with normal chicken tissue or serum. Examination with the same serums employed with myeloblastosis revealed the same phenomenon in like degree with the virus of erythroblastosis. In this respect, then, the 2 agents, each responsible for specifically different forms of avian leukemia, were at least qualitatively similar. No inference could be drawn relative to quantitative estimates or differences, as will be mentioned later.

A striking serologic difference between the viruses of myeloblastosis and erythroblastosis was evident, however, in the apparent lack of reaction of the latter agent with heterophile antibody. Antibodies induced in rabbits with homogenates of guinea-pig kidney were quite effective, though not so much as rabbit antibodies against normal chicken material, in the neutralization of myeloblastosis virus. The same serum (as well as a fresh preparation of anti-guinea-pig-tissue rabbit immune serum) had absolutely no effect on erythroblastosis virus in high dilution. The difference between the 2 agents, which is clear from comparison of text-figure 13 of (*1*) with the present text-figure 4, was quantitatively far greater than might be judged on superficial examination. The individual inoculums of myeloblastosis virus previously employed (*1*) contained 4.7×10^9 virus particles, whereas those of erythroblastosis, used with the same volumes of the same rabbit serum, contained only about 7.2×10^6 particles, an approximately 500-fold difference. This may or may not constitute a real difference, however, since the respective inoculums of both agents

were of comparable *levels of effect* on the host. The observations, within the experimental conditions, regardless of the underlying mechanisms and relationships, represented, nevertheless, an unequivocal distinction between the agents.

Interpretations of the earlier experiments with myeloblastosis led to the judgment that the virus contains at least 3 antigens—one specific to the agent *per se* and active in the homologous host, another identical with some component of normal host material, and a third of the nature of Forssman antigen. Since the respective antiserums were active in the neutralization phenomenon, it was inferred, further, that the antigens were firmly incorporated within that part of the virus particle involved in the specific capacity to exert infectious activity. The same interpretations, except those pertaining to Forssman antigen, are equally applicable to the virus of erythroblastosis. The results call to mind the earlier findings (*33,34*) with chicken sarcoma virus, which could be neutralized by exposure to antinormal-chicken-tissue immune bodies from the rabbit, *but only in the presence of complement*. Although this is a wholly different phenomenon from that observed with the leukemia viruses, which resulted from exposure to heated serum after absorption of heterophile antibodies with sheep red blood cells, it is pertinent to call attention to recent work by Rubin (*34*). This investigator, employing the system of titration on chick-embryo chorio-allantoic membrane, concluded from his results that the immune serum acted directly—not on the virus *in vitro* but on the susceptible host cells *in vivo*. This author implied that the mechanism involved in the problem of the leukemia viruses might be the same as that (whatever its actual nature) concerned with the Rous sarcoma virus. It must be emphasized that Rubin's work with the sarcoma agent differed from that with the leukemia viruses in the principle of the reaction, in the conditions and techniques of the experiments, and, most important of all, in the virus studied. There is no basis whatever for comparisons of the results with the different virus systems and, consequently, none for speculations about interpretations of Rubin's findings as they might apply to the leukemia viruses. A solution can be effected only by analogous experiments with the agents of erythroblastosis and myeloblastosis. Such studies are already under way.

In the cross-serologic reactions with antibodies from the homologous host, *the chicken*, there were qualitative similarities between the agents in the neutralization reaction. This was of more significance than the neutralization reaction with antimyeloblastosis *rabbit immune serum*, a complex system at best, in showing relationships between what might be regarded, for want of a more meaningful term, specific viral antigens. It should be stressed, however, that the design of the experiments, which involved large differences between the virus-particle content of the inoculums of the respective agents, as well as unknown differences in the antibody content (protein-antibody concentration) of the corresponding immune serums, can permit no interpretations relative to the quantitative aspects of the problem. This is not really of paramount importance at

the moment, however, since other properties serve effectively to establish interpretable distinctions between the agents.

Much was learned from the cross-precipitin reactions *with chicken serum*. Here, there was unquestionable evidence of difference in specific viral antigenicity. Antimyeloblastosis chicken immune serum, which precipitated completely the virus of myeloblastosis, induced but partial precipitation of the virus of erythroblastosis, despite repeated trials. In the same direction antierythroblastosis immune serum partially but definitely precipitating erythroblastosis virus, had little, if any, effect on the same myeloblastosis-virus preparation, which was completely precipitated by antimyeloblastosis immune serum. This certainly constitutes quantitative evidence of antigenic difference.

From all of the data gathered in the present work, in the immunologic studies on myeloblastosis reported before, and in experiments still to be described, it is now clear that myeloblastosis and erythroblastosis are not caused by the same agent but by unquestionably distinct though related viruses. In view of the various data comparing the viruses in this laboratory that show similarities as well as differences in host response (*3,20*), host-age differences in reaction and susceptibility (*3,14*), and the low, or absent, adenosinetriphosphatase activity in the virus of erythroblastosis (*3,35*) [exhibited by the agent of myeloblastosis (*16,17*)], as well as the numerous accounts (*3,4,9*) of the specific pathologic characteristics of the 2 diseases, there is little to be gained by detailed consideration of the literature. The various speculations about the etiologic relationships of the leukemias have been set down in several reviews (*4,36–39*), which cite the pertinent reports. The principal point is that these tumor agents, while related, are not the same, which clarifies one segment of a major problem of the leukosis complex. It might be speculated that the 2 agents are variants of each other and that each might be transformable in nature to the other. Observation of frequent mixtures of the diseases in which one or the other may predominate in different hosts might be explainable as due to initial mixture of the viruses. From the point of view of the whole picture of the leukosis complex, it would appear somewhat more reasonable, for purposes of future experiments, to place more emphasis on the possibility of variation and transformation and to regard the 2 viruses as intervariants, probably derived originally by conditioning and selection from some common stem agent. There is already some evidence, obtained in collaborative work with Burmester (Regional Poultry Research Laboratory, East Lansing, Mich.) and this laboratory, that the latter possibly is more likely to be the case than that the phenomena of leukosis as a whole are related to mixtures of different and/or related viruses.

There is one further point, introduced in an earlier report (*14*), deserving mention. The ultimate factors concerned with resistance and susceptibility to infection and the induction of detectable and progressive neoplasia by the tumor viruses remain obscure. This is of outstanding importance, especially in the highly conditioned mouse leukemia and

mouse mammary carcinoma. One influence often considered is that of some as yet unrecognized antibody or antibody-like substance. That a basis for speculations about such factors relative to the chicken leukemias is dubious, or possibly wholly lacking, can be concluded by the difference of host-age influence in the 2 diseases. Young chicks are most susceptible to myeloblastosis; older ones, at least to 77 days of age, are more susceptible to erythroblastosis than 3-day-old birds. Since the agents are closely related antigenically, it would be expected that hypothetical antibodies or antiviral substance would be equally effective with both at any given age of the chicken. Such, obviously, is not the case. It seems apparent, instead, that the immediate site of response is that of the host cell *per se* and that resistance or susceptibility is vested in the finest elements of host-cell constitution and physiologic state. Thus, it would appear that, although many influences may be in operation such as age, nutrition, sex, and hormones, to mention a few, the final problem devolves to consideration of the intimate genetic and constitutional status of the potential host cell involved and not to the organism as a whole.

Summary

Concentrates of the virus of an apparently pure strain of erythroblastosis have been obtained from large volumes of plasma and, after inactivation with formaldehyde, have been employed for the immunization of chickens. The resulting blood-serum antibodies effectively neutralized and gave precipitin tests with the virus.

The virus of erythroblastosis was neutralized by antiserum from rabbits inoculated with homogenates of normal chicken tissue and normal chicken serum. These reactions, similar to those obtained with the virus of myeloblastosis, were interpreted as indicating the presence, in the particles of erythroblastosis virus, of components antigenically similar to chicken protein intrinsically incorporated in the moiety of the particles responsible for infectious activity. In this respect the virus of erythroblastosis was closely similar to that of myeloblastosis, described in previous reports. On the other hand, the virus of erythroblastosis differed from myeloblastosis in the failure of the virus to respond to Forssman antibody.

Cross-serologic tests showed that the viruses of myeloblastosis and erythroblastosis were antigenically similar in reciprocal neutralization tests with chicken serum and that the agent of erythroblastosis was very effectively neutralized by antimyeloblastosis immune serum from the rabbit. In reciprocal precipitation tests, however, a difference was evident. Antimyeloblastosis chicken immune serum precipitated completely the virus of myeloblastosis but only partially the agent of erythroblastosis. Antierythroblastosis immune serum caused partial precipitation of erythroblastosis virus but had little or no effect on the same preparation of myeloblastosis virus precipitable completely by antimyeloblastosis immune serum. From these and other experiments, it has been concluded that the agents of myeloblastosis and erythroblastosis are distinct though closely related biologic entities. Implications of the

results concerned with possible relationships between these viruses with respect to intervariations or derivation from hypothetical stem agents were discussed.

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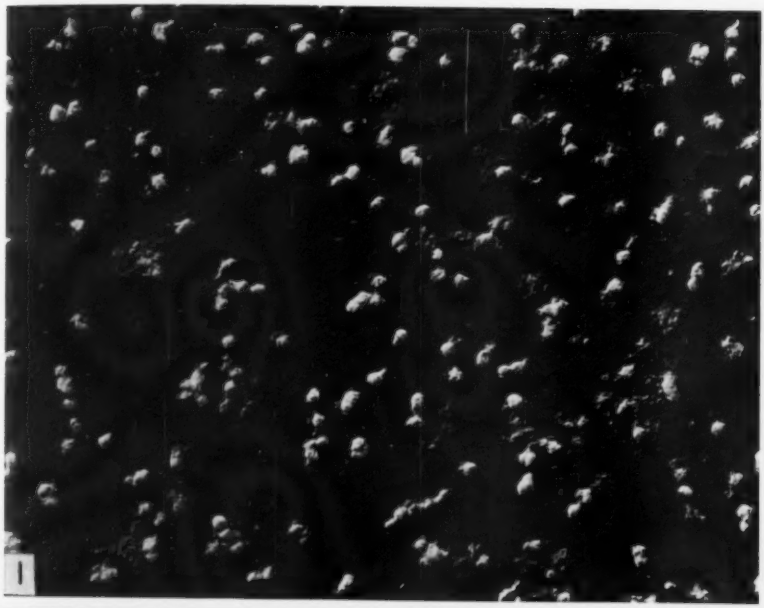
PLATES

PLATE 26

FIGURE 1.—Concentrate of erythroblastosis virus. × 27,000

FIGURE 2.—Specific aggregation of particles in a concentrate of erythroblastosis virus treated with antierythroblastosis immune serum from the chicken. × 27,000



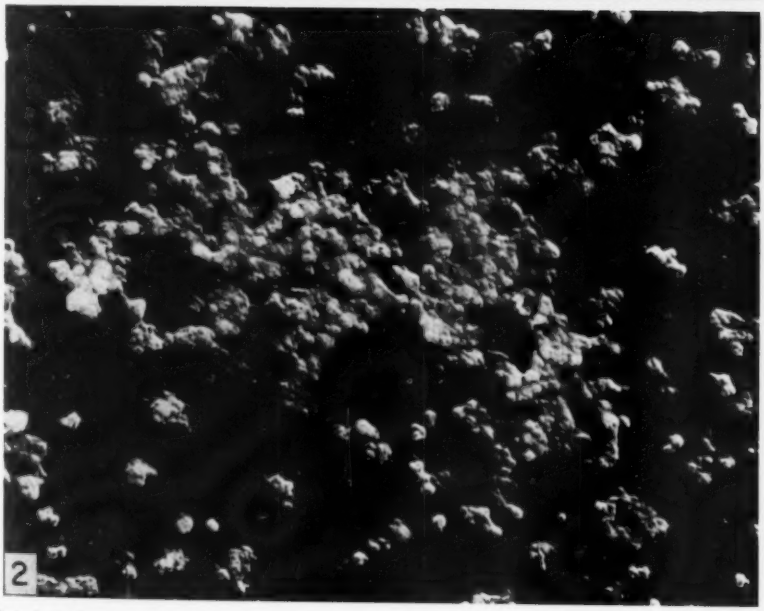


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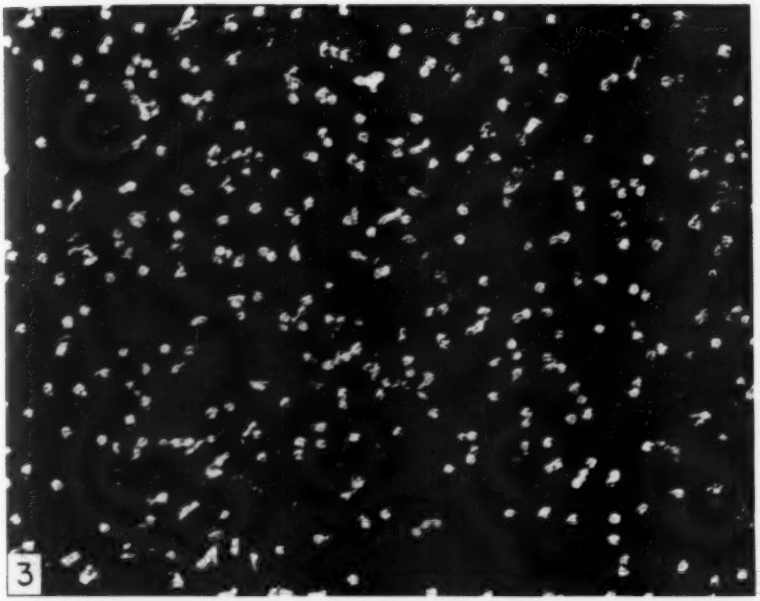
PLATE 27

FIGURE 3.—Virus of myeloblastosis in filtered plasma. × 21,000

FIGURE 4.—Specific aggregation of the virus of myeloblastosis in filtered plasma after treatment with antimyeloblastosis immune serum from the chicken. × 21,000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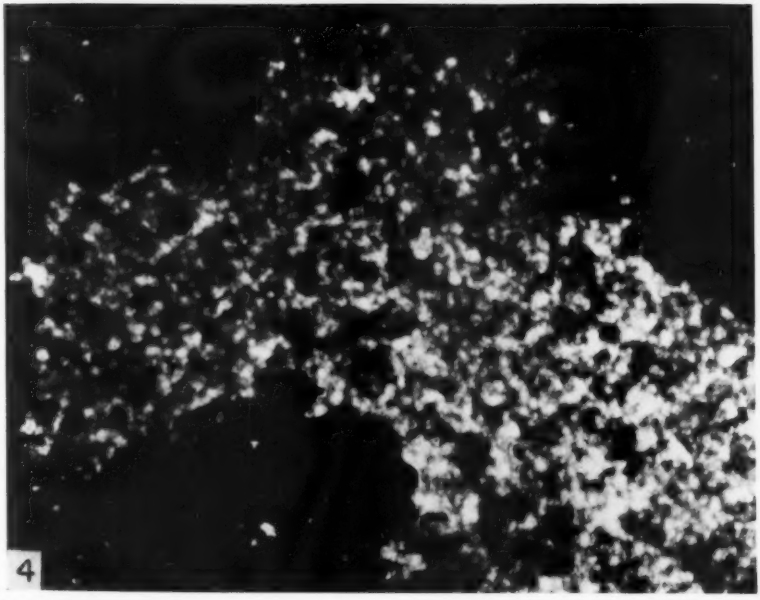
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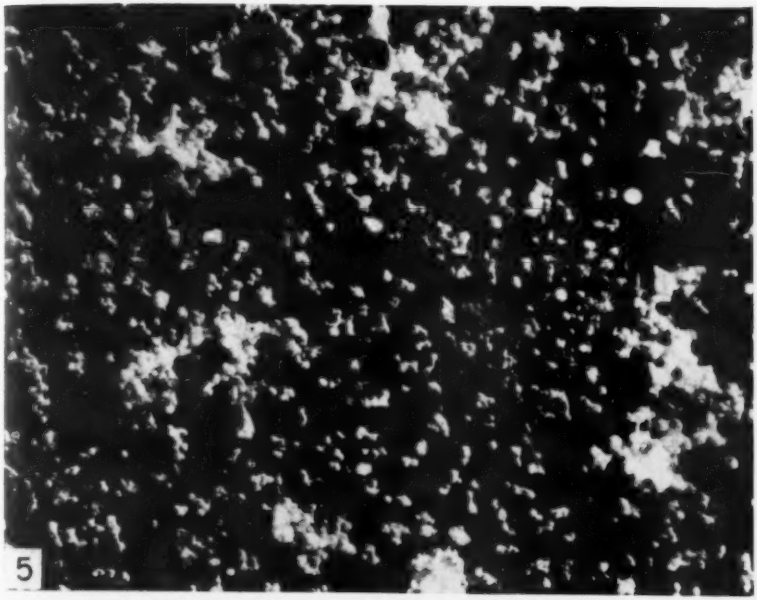
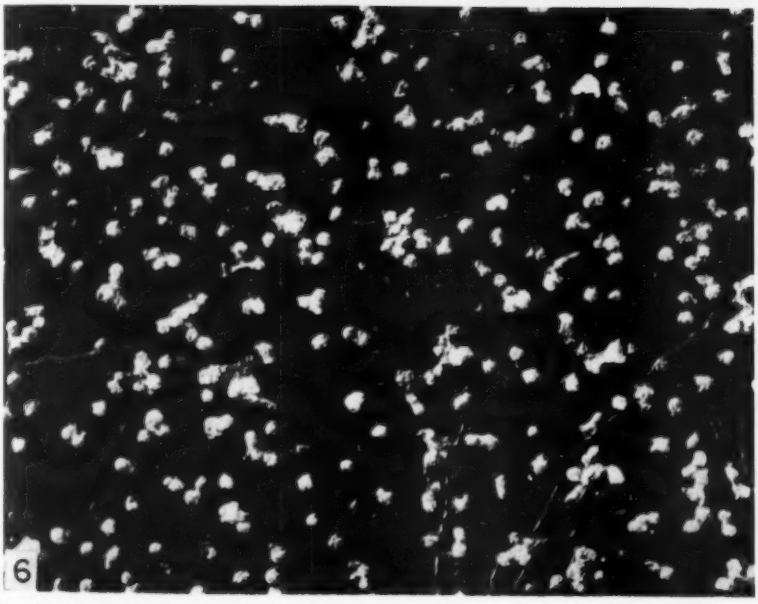
PLATE 28

FIGURE 5.—Partial aggregation of particles in a concentrate of erythroblastosis virus treated with antimyeloblastosis immune serum from the chicken. × 21,000

FIGURE 6.—Questionable aggregation of the virus of myeloblastosis in filtered plasma treated with antierythroblastosis immune serum from the chicken. × 21,000





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Choline-Oxidase Activity of L4946 Leukemic Cells of Mice [1]

BERNARD SHACTER, *National Cancer Institute,*[2] *Bethesda, Maryland*

Choline oxidase, an enzyme system oxidizing choline to betaine, occurs abundantly in the livers and kidneys of many species (*1,2*). Dinning *et al.* (*3*) found that feeding the folic acid antagonist aminopterin to monkeys resulted in almost complete disappearance of choline-oxidase activity from these organs. They suggested that folic acid functions in the choline-oxidase enzyme system, possibly as a constituent of the prosthetic group. Subsequent studies by several investigators, notably Williams, strengthened the concept of a relationship between choline oxidase and the folic acid group of compounds [3] (*1,4*). Dinning *et al.* (*5,6*) made the important finding that choline oxidase not only occurs in chick bone marrow, a tissue profoundly affected by folic acid antagonists, but that the enzyme disappears completely in folic acid deficiency and following feeding of aminopterin.

These observations prompted a search for choline-oxidase activity in neoplastic cells affected by folic acid antagonists. The present report describes the occurrence and some properties of choline oxidase in lymphocytic leukemia L4946, an experimental neoplasm of mice, growth of which is markedly retarded by amethopterin (4-amino-N^{10}-methyl-pteroylglutamic acid) (*7*).

Materials and Methods

The neoplasm was grown in ascitic form in (C3Hf × AKR)F_1 hybrid mice, as previously described (*7*). Fluids of several mice, withdrawn on the 7th post-transplant day, were pooled to supply sufficient cells for each experiment. The chilled [4] suspension was centrifuged, the supernatant fluid was discarded, and 0.25 *M* sucrose was added to give an approximately 15 percent suspension of cells. An aliquot of the uniformly suspended cells was added to a Wintrobe hematocrit tube for determination of the packed volume of cells; the remainder was homogenized in a pestle homogenizer for 4 minutes. Microscopic examination revealed few, if any, unbroken cells.

[1] Received for publication September 25, 1956.
[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.
[3] Pteroylglutamic acid, citrovorum factor, and related compounds.
[4] Unless otherwise indicated, all subsequent manipulations were carried out in the cold.

Most enzymatic determinations were carried out on a residue of the whole homogenate, prepared as follows: The homogenate was centrifuged at 10,000 × g for 15 minutes in the high-speed head of the International PR-2 centrifuge. The supernatant opalescent fluid, designated as the supernatant (S) fraction, was carefully pipetted off and replaced with an equal volume of distilled water. The residue was then uniformly suspended by gentle rehomogenization to give the rather viscous nuclear plus mitochondrial (N + M) fraction.

A mitochondrial (M) fraction was prepared by initial centrifugation of the homogenate at 700 × g for 10 minutes. The supernatant mitochondrial layer was pipetted off and subjected to centrifugation for 15 minutes at 10,000 × g, removal of the supernatant fluid, and resuspension of the residue in water as in the preparation of the N + M fraction.

Since choline oxidase has been investigated most extensively in liver, the content and distribution of enzyme in leukemic cells was compared with that in livers of normal (C3Hf × AKR)F_1 hybrid mice. The livers were rapidly excised, chilled, and homogenized for 2 minutes in 0.25 *M* sucrose to give a 1:10 homogenate. Fractions of liver were prepared as described for the leukemic cells.

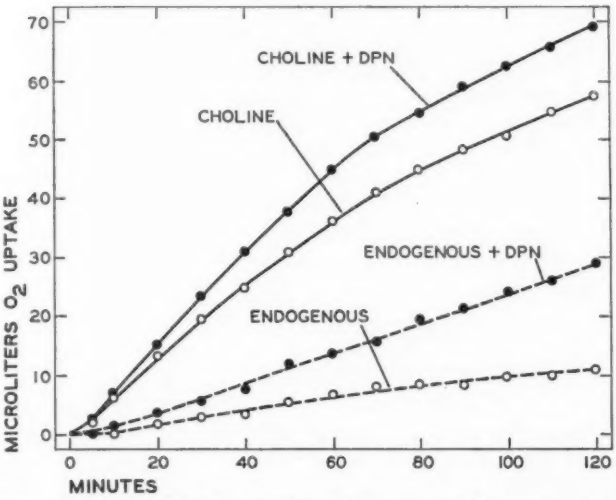
Choline-oxidase activity was determined by standard Warburg techniques. Unless otherwise indicated, main chambers of Warburg vessels contained 1.0 ml. of 0.1 *M* phosphate buffer, *p*H 7.3, 3 μM neutralized diphosphopyridine nucleotide (DPN), tissue suspension derived from 0.15 ml. packed leukemic cells or 0.1 gm. liver, and water to give a final volume of 3.0 ml. upon subsequent addition of sidearm contents. Side arms contained 5 mg. of choline chloride in phosphate buffer in experimental flasks or an equal volume of phosphate buffer in control flasks. Central wells contained 0.2 ml. of 10 percent KOH and fluted filter paper. The gas phase was air. Tissue preparations were added to Warburg vessels containing all other constituents, the vessels were equilibrated in the bath at 37° C. for 10 minutes, and the sidearm contents were tipped in. Readings were taken every 10 minutes, usually for 1 hour.

Nitrogen content of tissue preparations was determined by digestion and nesslerization, essentially as described by Umbreit *et al.* (*8*).

Endogenous uptake of oxygen in control flasks was subtracted from the total uptake in choline-containing flasks to give the net uptake due to enzymatic activity. Choline-oxidase activity was calculated as net O_2 uptake per hour per ml. of packed leukemic cells (or per gm. of liver), or as $Q_{O_2}(N)$, the net O_2 uptake per hour per mg. nitrogen.

Results and Discussion

Preliminary experiments demonstrated the presence of choline-oxidase activity in whole homogenates of the leukemic cells. Endogenous O_2 uptake, however, accounted for the major portion (about 70%) of total oxygen utilization. With elimination of endogenous substrate by high-speed centrifugation of the homogenate and removal of the supernatant fraction, the endogenous rate of O_2 uptake was decreased considerably (*cf. 9*). Text-figure 1 shows the choline-oxidase activity as well as the



TEXT-FIGURE 1.—Choline-oxiadse activity and endogenous O_2 uptake of the N + M fraction of leukemic cells. Values shown with added choline are net uptakes after correction for endogenous oxygen utilization.

endogenous O_2 uptake of such preparations. Since DPN stimulates the choline-oxidase activity of residues of mouse-liver homogenates (*10*), the influence of the nucleotide on leukemic-cell preparations was determined.

There was a slight lag in choline-oxidase activity during the first 5 minutes. Activity then reached a maximum and, in the presence of DPN, remained relatively constant until about the end of the first hour, falling off slightly toward the end of the hour and more rapidly thereafter. In the absence of DPN a gradual decrease in enzymatic activity was evident throughout the 2-hour observation period. Endogenous O_2 uptake of unsupplemented preparations represented about 16 percent of the *total* uptake with choline added. Addition of DPN doubled the rate of endogenous O_2 uptake and increased choline-oxidase activity about 24 percent, the ratio of endogenous to total oxygen uptake becoming about 24 percent.

The choline-oxidase system of rat liver appears to be composed of 2 enzymes, choline dehydrogenase, oxidizing choline to betaine aldehyde, and betaine-aldehyde dehydrogenase, converting betaine aldehyde to betaine. There is uncertainty at present whether both enzymes or only the latter are DPN-linked (*1*). No attempt was made to separate possible component enzymes in the present work on leukemic cells. Since DPN stimulated the over-all enzymatic oxidation of choline, it was added in all of the following experiments.

Table 1 demonstrates the reproducibility of values for choline-oxidase activity of leukemic cells. The 10 consecutive determinations were carried out on 2 lines of the neoplasm over a 2-month period.[5]

[5] Lines A and B of L4946, ascitic transfer generations 100 to 109, were used.

TABLE 1.—*Choline-oxidase activity of leukemic cells*

Determination	Number of mice*	Choline oxidase†	Endogenous O_2 uptake (*percent total*)
1	11	323	23. 9
2	8	311	22. 4
3	8	299	30. 8
4	10	309	28. 9
5	10	312	25. 3
6	10	301	22. 1
7	10	357	21. 6
8	4	328	21. 0
9	5	302	24. 7
10	4	311	22. 2
Mean ± standard error		315 ± 5	24.3 ± 1.0

*Determinations, in duplicate, on pooled ascites cells from number of mice shown.

† Microliters O_2 per ml. cells per hour.

Kensler and Langemann (*11*), as well as Williams (*12*), found choline-oxidase activity of rat liver to be concentrated largely in mitochondria. This appeared to be true also for the leukemic-cell enzyme, the M fraction accounting for 74 percent of the total activity (table 2). Since the M fraction contained only about 24 percent of the nitrogen in the combined N + M fraction, the mitochondrial $Q_{O_2}(N)$ far exceeded that of the total residue.

TABLE 2.—*Choline-oxidase activity of fractions of leukemic cells and liver*

Preparation	Leukemic cells			Liver		
	Total nitrogen (*mg./ml.*)	Choline-oxidase activity		Total nitrogen (*mg./gm.*)	Choline-oxidase activity	
		Total*	$Q_{O_2}(N)$		Total†	$Q_{O_2}(N)$
Homogenate	29. 7	246	8. 3	32. 0	994	31. 1
N + M fraction	13. 7	323	23. 6	16. 6	876	52. 8
M fraction	3. 3	238	71. 2	9. 5	662	69. 7
N fraction‡	10. 4	85	8. 2			

*Microliters O_2 uptake per ml. cells per hour.

†Microliters O_2 uptake per gm. liver per hour.

‡Calculated as difference between N + M and M fractions.

Difficulty was encountered in separating a satisfactory nuclear preparation from the viscous nuclear fraction. As determined by the difference between the N + M and M fractions, however, the nuclear moiety showed little enzymatic activity. The S fraction of the leukemic cells, although containing approximately two thirds of the total cellular nitrogen, showed no choline-oxidase activity whatever. It seems not unlikely, therefore, that choline oxidase of the leukemic cells is localized entirely in the mitochondria.

Miller (*10*) found that a concentration of 5×10^{-3} *M* nicotinamide increases choline-oxidase activity of residues of mouse-liver homogenates. This was confirmed in the present work. Choline-oxidase activities of the homogenate and fractions of mouse liver were increased about 25 percent upon addition of nicotinamide to a final concentration of 5×10^{-3} *M*. This amount of reagent was therefore added in obtaining the values for liver choline oxidase shown in table 2.[6] Oxidation of choline fell off fairly rapidly after 30 minutes; calculation of choline-oxidase activity in liver was therefore based on oxygen uptake during the first 30 minutes.

Total activity of choline oxidase in the N + M fraction of mouse liver was about 2½ times greater than that in leukemic cells. Approximately 76 percent of liver choline-oxidase activity appeared localized in the M fraction, in agreement with values obtained for rat liver (*11,12*). Enzymatic activity in liver mitochondria per gm. of liver, therefore, far exceeded that of leukemic-cell mitochondria per ml. of packed cells.[7] The content of nitrogen in the mitochondrial fraction of leukemic cells, however, was about one third that in the liver fraction, and the $Q_{O_2}(N)$ of leukemic-cell mitochondria equaled that of liver.

The choline-oxidase activity of the N + M fraction of leukemic cells was invariably greater than that of the unfractionated homogenate. Williams (*12*) demonstrated the presence of a heat-labile inhibitor in the supernatant fraction of rat-liver homogenates, which inhibited choline-oxidase activity when added to mitochondria or mitochondria combined with nuclei. The inhibitor appeared to be released during the fractionation process, since unfractionated homogenates showed a choline-oxidase activity equivalent to that of the combined nuclear, mitochondrial, and microsomal fractions. In the present work also the unfractionated homogenate of mouse liver usually showed an enzymatic activity about equal to that of the N + M fraction. One possible explanation for the lowered enzymatic activity of leukemic-cell homogenates is that a soluble inhibitor is released into the homogenate even without fractionation. Langemann and Kensler (*13*), on the other hand, noted that the addition of succinate reduced the choline-oxidase activity of liver homogenates, presumably through saturation of proton and electron receptors present in the homogenates. It may be possible, therefore, that there is sufficient endogenous substrate present in whole homogenates of leukemic cells to inhibit choline-oxidase activity through a similar mechanism.

Barron and co-workers (*14,15*) have shown that rat-liver choline oxidase is strongly inhibited *in vitro* by nitrogen mustard (HN_2)[8] and by numerous thiol reagents. Experiments were carried out to determine the influence of nitrogen mustard and of *p*-chloromercuribenzoic acid on choline-oxidase activity of N + M fractions of leukemic cells. The reagents were added to the main chambers of Warburg vessels prior to the addition of the

[6] Nicotinamide had little, if any, effect on choline oxidase of leukemic-cell fractions and was therefore omitted in these determinations.

[7] On the basis of nitrogen content, 1 ml. of packed leukemic cells was approximately equivalent to 1 gm. wet weight of liver.

[8] Methyl-bis(β-chloroethyl)amine.

TABLE 3.—*Influence of inhibitors on choline-oxidase activity of leukemic cells*

Concentration (*M*)	Nitrogen mustard		*p*-Chloromercuribenzoic acid	
	Choline oxidase*	Inhibition (*percent*)	Choline oxidase*	Inhibition (*percent*)
0	312	—	311	—
3.3×10^{-6}	294	6	—	—
1.0×10^{-5}	259	17	285	8
3.3×10^{-5}	179	43	226	27
1.0×10^{-4}	69	78	143	54
3.3×10^{-4}	0	100	0	100

* Microliters O_2 per ml. cells per hour.

tissue preparations. Immediately upon addition of the latter, the flasks were placed in the bath, equilibrated for 10 minutes, and the sidearm contents were tipped in. From table 3 it is apparent that the 2 reagents are potent inhibitors of leukemic-cell choline oxidase.

The foregoing data indicate the occurrence, in an experimental lymphocytic leukemia, of a choline oxidase having properties similar to that of the enzyme found in liver. Experiments are under way to determine whether suppression of growth of the neoplasm by folic acid antagonists is associated with inhibition of the enzyme.

Summary

An enzyme showing choline-oxidase activity is present in ascitic cells of the experimental lymphocytic leukemia L4946. The activity of the enzyme *in vitro* is enhanced by diphosphopyridine nucleotide and markedly inhibited by nitrogen mustard and by *p*-chloromercuribenzoic acid. The enzyme is localized mainly in the mitochondrial fraction of the leukemic cells, its activity per mg. nitrogen equaling that of mouse-liver mitochondria.

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Evaluation of Cancer Diagnostic Tests

◆A program for evaluating proposed cancer diagnostic tests, primarily from the standpoint of usefulness for case finding in the general population, was inaugurated by the National Cancer Institute in 1948. A methodology and criteria for such evaluation were proposed by J. E. Dunn, Jr. and S.W. Greenhouse (Cancer Diagnostic Tests. Principles and Criteria for Development and Evaluation. Washington, D. C., U. S. Public Health Service, Publication No. 9, 1950, 23 pp.) and the program was implemented by special project grants awarded on recommendation of the National Advisory Cancer Council.

◆In 1950, a conference on cancer diagnostic tests was held and its proceedings were published (Proceedings of the First Conference on Cancer Diagnostic Tests. Sponsored by the National Advisory Cancer Council. Washington, D. C., U. S. Public Health Service, Publication No. 96, 1951, 91 pp.). In 1953, a compilation of reports of work performed under the program appeared as a monograph (Evaluation of Cancer Diagnostic Tests. Washington, D. C., U. S. Department of Health, Education, and Welfare, Public Health Service, Public Health Monograph No. 12, 1953, 49 pp.).

◆The present collection of 9 papers is another group of results of work performed under the program. This records further research effort directed toward an important goal. However, a test suitable for cancer case finding in the general population still remains to be found.

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Infrared Spectroscopy of Crystalline Albumin in Human Neoplasia [1, 2]

CONRAD T. O. FONG,[3] STUART W. LIPPINCOTT,[3] *and* NILS ERIKSEN,[4] *Field Investigations and Demonstrations Branch, National Cancer Institute,*[5] *and Department of Pathology, School of Medicine, University of Washington, Seattle, Washington*

A systematic study of the concentrations and characteristics of the serum proteins in neoplastic diseases is being carried out in this laboratory. This report deals with the infrared absorption spectra of human crystalline albumin prepared from the serums of normal individuals, from several with miscellaneous non-neoplastic diseases, and from 12 patients with carcinoma from 6 different sites of origin. The interaction of infrared rays and matter can be recorded as curves on coordinate paper, plotting wavelength, in microns, *versus* absorption as percent transmission, and many types of bonds have a maximum absorption at particular wavelengths that are characteristic of them. In this way functional groups and linkages of various materials have been identified. For example, this technique has been used for the identification of some sugars and their derivatives, to distinguish the serologic relationship of pneumococcal polysaccharides and certain of the linkages for some native globular protein. The absorption spectrum of bovine serum albumin has been studied (*1*) and compared with a synthetic polypeptide (polylysine) to determine the identity and characteristics of certain groups. In our work we took cognizance of these fundamental observations that others had reported, chiefly those on the N-H and C-O vibrations of the amides. The objective of our investigation was to determine whether there is any alteration in the albumin of patients with neoplastic diseases that could be used to distinguish it clearly from that of normal individuals.

Materials and Methods

Fresh serums were obtained from blood drawn from the donors during the postabsorptive state. Individual specimens and pooled serums were used from the normal controls and individual specimens only in the miscellaneous and neoplastic cases. The latter were selected to include 9

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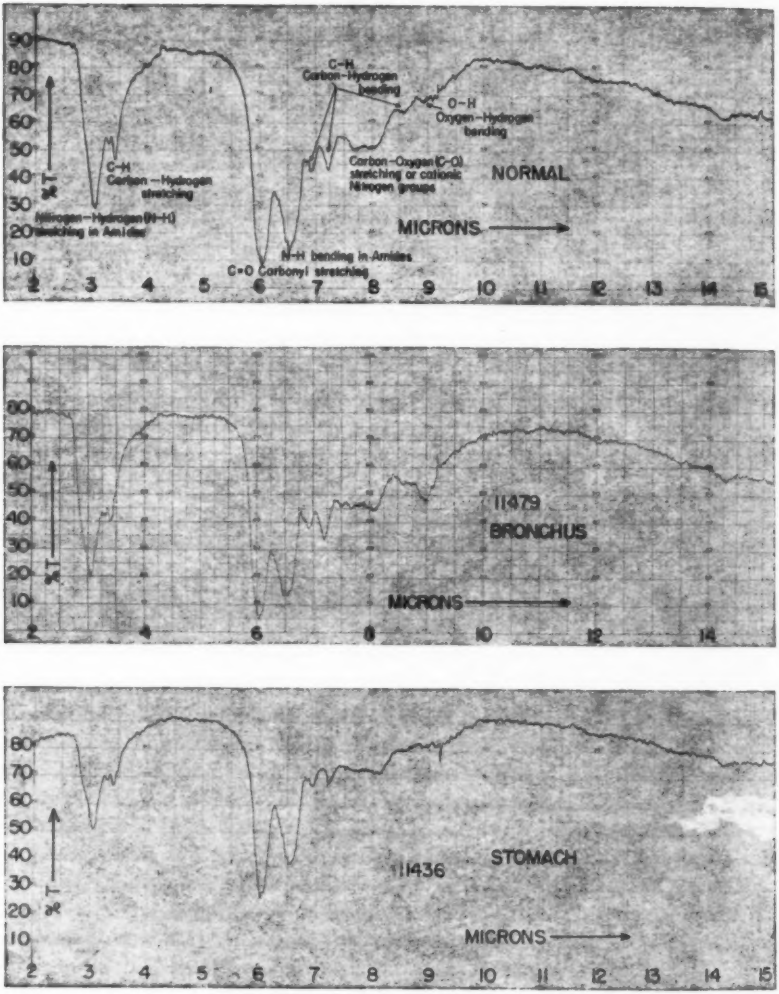
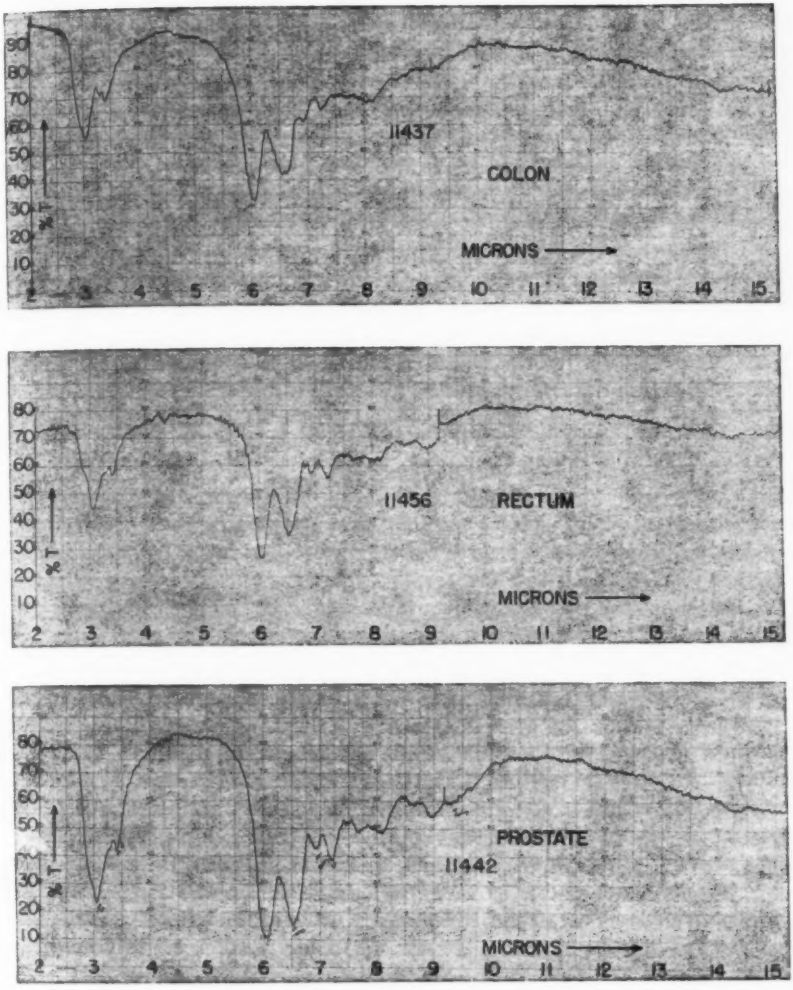
[2] Presented in part at the meeting of the American Association for Cancer Research.
[3] *Present address:* Medical Dept., Brookhaven National Laboratory, Upton, Long Island, N. Y.
[4] Dept. of Pathology, University of Washington.
[5] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.



TEXT-FIGURE 1.—Infrared spectra of albumin from serums



of a normal pool and 5 histologic types of carcinoma.

cases prior to any form of treatment and 3 cases in which previous irradiation or surgery had been employed but residual disease was still present. In the group studied prior to treatment 5 had known metastases. The sites of origin of the malignant lesions were tongue, stomach, descending colon, rectum, bronchus, and prostate. All the diagnoses were established by microscopic examination of tissue from the lesions.

Electrophoretic analyses of the crystalline albumin from normal individuals and from those with various types of cancer were carried out in the Pearson model of the Tiselius apparatus under constant conditions; namely, barbital buffer, ionic strength 0.1, *p*H 8.5 to 8.6; bath temperature, 0° C.; field strength, 8.9 to 9.0 volts per centimeter; time of migration, about 2 hours; protein concentration, 0.7 to 0.9 gm. per 100 ml. The albumins from the 2 sources had identical mobilities and accounted for approximately 90 percent of the total material present. There was no indication, under these conditions, of any qualitative differences among the various albumins, regardless of origin.

In the fractionation of the serums part of Cohn's alcohol procedure was utilized (*2*). This procedure consisted of diluting the serum to an ionic strength of 0.09, adjusting the *p*H to 5.8, and adding cold 95 percent ethanol dropwise with stirring to the diluted serums until a concentration of 40 percent ethanol was reached. The temperature was kept several degrees above the freezing point of the alcohol solution, except when a 40 percent ethanol concentration was reached; at this point the temperature was maintained at −10° C. The precipitate formed at this *p*H and alcohol concentration consisted mainly of globulins, which were removed by centrifugation. By lowering the *p*H of the supernatant liquor to 4.8 the albumin was precipitated and collected by centrifugation. Hughes' method (*3*) was then utilized to purify the albumin further. The process involved the solution and precipitation of albumin with alcohol in the presence of cysteine, a cycle of solution and precipitation of the albumin with alcohol, and repeated suspension of the albumin in cold alcohol with various ionic strengths of acetate buffer. The mercury dimer of the albumin was prepared by the addition of mercuric chloride. Crystallization took several weeks. The mercury dimer, after 1 recrystallization, was repeatedly washed with cold 15 percent ethanol, centrifuged, dissolved in 0.01 *M* cysteine, and dialyzed against water. The purified albumin was lyophilized; the yield was generally rather low.

The spectral studies of the albumin and dried serums were carried out in the solid state in potassium bromide discs. To prepare a sample, dry, finely powdered potassium bromide was mixed and ground with the lyophilized protein in an agate mortar. The powder was then poured into a die, placed in a hydraulic press, and the die was subjected to a pressure of approximately 20 tons. The disc thus prepared was examined with a double-beam infrared spectrophotometer.

Results

Text-figure 1 presents the infrared absorption spectra obtained from the crystalline albumin of a representative normal control and from 5 typical cases with carcinoma of various sites of origin.

Bands were obtained at 3.05, 3.42, 6.04–6.10, 6.5–6.55, 6.95, 7.20, 7.70–8.15, 8.55–8.6, and 9.0μ. The probable assignments of frequencies are 3.05μ due to N-H stretching in amides, 3.42μ C-H stretching, 6.04–6.1μ carbonyl stretching, 6.5–6.55μ N-H bending in amide, 6.95, 7.20, 8.55–8.6μ C-H bending, 7.70–8.15μ C-O stretching or cationic nitrogen groups, and the 9.0μ due to O-H bending, although this is shifted to a somewhat lower wavelength. Since the assignments in the higher region are not definite no attempt was made to correlate the bands observed.

Summary and Conclusion

The infrared spectra of crystalline albumin prepared from the serums of normal controls and from those of 12 patients with carcinoma from 6 sites of origin showed essentially no differences in the regions where the assignments are definite.

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A Study of the Penn-Hall Serofloc-culation Reaction for Cancer [1,2]

ANDREW C. PEACOCK *and* GEORGE Z. WILLIAMS,
Clinical Trials Unit, National Cancer Institute,[3]
and Clinical Pathology Department, Clinical Center,
Bethesda, Maryland

In a recent paper, Penn and Hokama (*1*) have summarized some of their work on the "antigenic" properties of certain bile-acid derivatives, particularly ethyl choladienate. These derivatives are reported to have the property of producing a flocculation reaction when mixed with the serum of cancer patients. Hall, Dowdy, Penn, and Bellamy (*2*) have made a study of this reaction in a large series of diverse serums, including many from cancer patients. Their results indicated that the flocculation reaction rarely occurred in serums from normal persons, or persons with diseases other than cancer, while almost all from patients with invasive cancer produced flocculation.

The present study is an evaluation of the suggestion of Hall *et al.* that the serofloculation reaction using ethyl choladienate might find use as an "adjunct to the detection of cancer" (*2*). We have limited our study to the problem of whether or not another laboratory, following exactly the directions of Penn and Hokama, can in fact obtain the same high degree of sensitivity and selectivity described by them. As a part of this investigation we have studied the reproducibility of the test, because information on this point was not presented in the cited publications.

Method

The "qualitative seroflocculation reaction" described by Penn and Hokama (*1*) employs an antigen suspension which is prepared by adding a buffered sodium chloride solution to an alcoholic solution of cholesterol and the antigen ethyl choladienate. Two tubes are used for each test. One contains 0.03 ml. serum (inactivated at 56° C.), the other, 0.02 ml. Antigen suspension (0.075 ml.) is added to each tube. The tubes are shaken in a mechanical shaker, 1 ml. of 0.6 percent sodium chloride solution is added, and the tubes are centrifuged. The resulting precipitate is dispersed by tapping the tube gently. If the particles remain small and are suspended in a turbid medium the reading is "negative." If there is

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flocculation, producing larger particles in a "sparkling" clear medium, the reading is "positive." Doubtful cases are recorded as "negative." A test is positive if either tube is positive. Details are described in the original report (*1*).

In order to insure detailed conformity with this procedure in our laboratory, the following steps were taken:

1) A senior technician visited Dr. Hall's laboratory and received detailed personal instruction.

2) Dr. Hall clarified several points in the instructions which were ambiguous to us.

3) Samples were exchanged between his and our laboratories. Considering the errors inherent in the procedure (*see* below) our results were in good agreement.

4) Dr. Penn and Mr. Hokama visited our laboratories and found no deviation in our procedure from their published instructions.

5) The antigens used in this evaluation were samples of ethyl choladienate prepared by Lederle Laboratories and supplied by Dr. Hall.

Reproducibility of Seroflocculation Reaction

Procedure

In order to assess the reproducibility of the Penn seroflocculation reaction we performed replicate assays on serums from each of 20 normal, healthy subjects[4] and later repeated the series of replicate experiments on a second sample of serum drawn from the same subjects. The second sample was obtained 10 days after the first.

For this part of the study only, 1 tube (0.03 ml. serum) was used. The replications were started with a fresh antigen suspension for each group, but only 1 lot of antigen was used; all other solutions were constant throughout. The replications, therefore, reflect variability in preparation of the antigen suspension, addition and mixing of components, the reaction itself, and the reading of the result.

Appearance of Dispersed Precipitate

Although for practical purposes a result may be recorded as negative if the flocculation is not complete, an assortment of serums may be easily arranged in a series in which the particle size varies continuously from the turbid negative to the large-particle positive. Thus, many serums which must be classified as negative, according to Penn and Hokama, differ with respect to particle size and turbidity of the medium. In order to indicate the observation that some serums produced flocculation that was of an intermediate character, we read flocculations in this ill-defined range as doubtful, but have tabulated them as negative in conformity with Penn's practice.

Replicate Experiments

Table 1 includes the pooled data from 2 different samples of serum from each of 20 normal subjects. Five were remarkable in that the readings

[4] Included as part of group 5, Evaluation section.

for the first and second samples were opposite, *i.e.*, positive in the first and negative in the second, or *vice versa*, table 2.

TABLE 1.—*Replicate tests for the Penn seroflocculation reaction on sera from 20 normal subjects**

Serum no.	Result		Serum no.	Result		Serum no.	Result		Serum no.	Result	
	No. +	No. −		No. +	No. −		No. +	No. −		No. +	No. −
1	0	29	6	1	18	11	4	25	16	13	8
2	0	14	7	1	13	12	5	24	17	19	9
3	0	14	8	1	18	13	6	8	18	19	0
4	0	14	9	2	17	14	7	27	19	25	5
5	0	14	10	3	25	15	12	16	20	29	0

*Two samples of blood drawn 10 days apart were replicated on each occasion. Total number of positive and negative results found on each person are listed in order of increasing number of positive results.

TABLE 2.—*Summary of test results on 2 specimens of serum from 5 of 20 normal persons shown in table 1**

Serum no.	Serum sample 11/4/54		Serum sample 11/14/54	
	No. +	No. −	No. +	No. −
13	0	4	6	4
14	2	22	5	5
15	11	7	1	9
16	13	4	0	4
17	17	2	2	7

* These serums, drawn 10 days apart, yielded conflicting results.

The reproducibility of the flocculation was not the same for all the serums studied. In 10 of the 20 serums (#1–8, 18, 20, tables 1 and 2) replicates were nearly constant, but in the remaining 10 serums (#9–17, 19) the variability was pronounced. In addition to variability, these 2 groups differed markedly with respect to the number of results originally recorded as doubtful. In the low-variability group only 2 percent of the 185 readings (table 1) were doubtful, but in the high-variability group 25 percent (of 259 readings) were doubtful.

Although there may be several causes for erratic behavior of this flocculation reaction in normal serums, the variability is not due to inaccurate reading of the test result. Despite the subjective nature of the end point, readings obtained after rearrangement of the tubes in the rack were in excellent agreement with those obtained earlier. Furthermore, independent readings by several laboratory personnel were in good agreement.

Evaluation for Cancer Detection

Procedure

Five lots of antigen (ethyl choladienate), identified as lots #1 to 5, were supplied by Dr. Hall.

Three hundred and thirty-one normal subjects were studied. Two hundred and ninety-seven (tested with antigen lots #1–4) were professional donors to the Clinical Center (National Institutes of Health) Blood Bank. These people were all carefully screened by a physician prior to the performance of the test and had no history of chronic disease or present illness. In each case the blood was judged suitable for transfusion. The other 34 were normal, healthy persons—24 laboratory personnel and 10 applicants for employment who had had a thorough medical examination. In addition to the qualitative seroflocculation reaction, all samples in groups 2 and 3 that were positive were studied further with serial dilution. This technique was not described in detail in reference (*2*) but protocols for the method were kindly furnished by Dr. Hall.

Results

The results of the tests on 331 normal individuals are given in table 3. Although the percentages vary somewhat with different antigens, there was no less than 35 percent positive in any one group of these normals; the over-all percentage of false positive results was 55 percent. Sixty-five positive serums from groups 2 and 3 were retested by the serial-dilution scheme, yielding the results in table 4. Twenty-seven percent were positive at dilutions of 1:24 or greater.

TABLE 3.—*Summary of test results on normal people*

Series	1	2	3	4	5	Total
No. persons	31	55	29	182	34	331
Percent positive	35. 5	72. 9	86. 2	52. 1	35. 3	55. 3

TABLE 4.—*Results of tests, including serial dilution, on 84 persons in series 2 and 3*

	Results							
	Neg.	*	Positive at each of following dilutions:					
			1:6	1:8	1:12	1:16	1:20	1:24 or greater
No. cases	19	14	7	5	5	6	5	23

*Fourteen persons, + on 2-tube assay, gave no further positives on dilution.

Discussion

The observation that flocculation in the ethyl choladienate-cholesterol-serum system varies in a continuous fashion from no flocculation to (apparently) complete flocculation implies a quantitative, rather than a qualitative, difference among various serums. For discussion only we define this property of serum to produce a flocculation as a "flocculation potential" without suggestion as to the actual factors involved. The quantitative (as contrasted to qualitative, disease-specific) nature of this flocculation potential is supported by the findings of Hall *et al.* that serums which initially are positive (*i.e.*, possess a high flocculation potential) become progressively inactive with sufficient dilution.

The extent to which a serum of given flocculation potential flocculates depends on the test conditions. Evidence for this is found in the observation [5] that *all* serums, under certain conditions, are positive, while with other modifications, many serums positive by the present technique are negative.[6] Factors which alter the sensitivity of the system include changes in purity of the antigen and the amount of cholesterol (*1*). Thus, in addition to the flocculation potential of the serum, factors affecting the stability of the ethyl choladienate suspension are also important. A positive result, therefore, means that the flocculation potential of the serum tested is greater than some critical value, which value is a property of the whole test system.

Minor variations in a given set of test conditions will alter the reaction most in those serums of flocculation potential at or near the critical value. The results in the series of replicates indicate that a substantial number of our normal serums had flocculation potentials in this critical range and were either positive or negative depending upon slight unknown variations in test procedures. More extensive changes in the test system, such as might arise when antigen preparations of different purity are used or when the system is set up by another laboratory with different reagents, etc., will undoubtedly be accompanied by a corresponding change in the sensitivity of the flocculation system.

When the critical flocculation potential of a system is low, many serums will produce a positive result, but in a system with high critical potential, relatively few serums will be positive. Sprunt, for example, reports positive results in 61 percent of cancer patients, in 41 percent of patients with miscellaneous noncancerous diseases (*3*), and in less than 5 percent of normals.[7] We believe that our apparently contradictory results of positive findings in 100 percent of cancer patients and 55 percent of normals reflects mainly a difference in sensitivity of the test system as set up in the 2 laboratories. This very discrepancy emphasizes the lack of reliability for detection of a useful proportion of the cancer group.

The results reported by Hall *et al.* for normal subjects are copied in table 5. It is noteworthy that the percentage of false positives among

[5] Y. Hokama, personal communication.
[6] A. C. Peacock, unpublished observations.
[7] D. H. Sprunt, personal communication.

unscreened normals is much higher with antigen D than with earlier preparations. (Whether or not the earlier results reported were "corrected" by screening was not disclosed.) We cannot subscribe to the practice of re-examining false positive cases by clinical screening for the purpose of finding some reason why they should not be classified as normal. A similar re-examination was not done on patients with an initial "correct" diagnosis, normal or cancer. Therefore, only their results on unscreened normals are comparable to our data.

TABLE 5.—*Results on normal subjects**

Antigen	No. cases	Positive (*percent*)	Remarks
A	992	1. 1	
A	998	0. 9	
B	50	4. 0	
B+C	1521	2. 8	
D	103	3. 8	
D	316	9. 0	Unscreened normals
		4. 7	After history check
		3. 0	History check and personal interview
D†	240	22	Unscreened normals
		3	History check and personal interview

*Hall *et al.* (2).
† Personal communication from Dr. Hall.

We agree with Dunn and Greenhouse (*4*) that ". . . it is inconceivable that a [cancer screening] test giving more than 5 percent false positive values would have any practical value." For this reason, we believe that the difference between 22 percent and 55 percent false positives is unimportant but emphasize that the evidence from all 3 laboratories points to the failure of this reaction to discriminate adequately between serums of those with and without cancer.

Summary and Conclusions

A serum flocculation reaction reported to be useful as an adjunct in the detection of cancer has been studied with regard to the details of the technique and to the results found in the serums of normal and cancer patients.

Of serums from 331 normal subjects, 55 percent were falsely positive. It is concluded that the method is not sufficiently reproducible for reliability and that this reaction is not suitable for general use as a cancer screening test.

Addendum

Publication by Blossom, Moore, and Butt (*5*) of their findings with the Penn test appeared after this manuscript was completed. The data presented, more closely resembling those published by Sprunt than our own, led them to conclude that the reaction was not at present suitable for use in cancer detection.

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On the Fuchs Cancer Test: Species-Specific Proteolysis of Fibrin by Serum [1]

ANDREW C. PEACOCK *and* ELEANOR M. HIGHSMITH,[2]
Clinical Trials Unit, National Cancer Institute,[3]
Bethesda, Maryland

Fuchs (*1*) reported studies of a reaction occurring when fibrin and blood serum are incubated together. He claimed that when the fibrin and serum were obtained, each from a different species (*e.g.*, horse fibrin and bovine serum), there was a substantial increase in the dialyzable nitrogen, but if fibrin and serum were from the same species, no such nitrogen liberation took place. He believed that the reaction was due to a species-specific proteolytic enzyme in the serum, which caused the breakdown of heterologous fibrin, releasing dialyzable nitrogen to the medium. Subsequently, Fuchs published data (*2*) indicating that a specific enzyme was present in the serums of cancer patients that differed from the enzyme in the serums of healthy people in a way analogous to the difference found in animal species studied previously, *i.e.*, cancer serum liberates nitrogen from normal fibrin but not from cancer fibrin, and *vice versa*. He claimed that the specificity of this reaction served to distinguish cancer from normal serums in more than 90 percent of the cases tested. Other laboratories investigated the utility of the Fuchs reaction for the diagnosis of cancer and, although a few (*3,4*) found the reaction to be of no value, at least 12 others, after investigating a total of 1700 cases, confirmed Fuchs' reports [*see* (*4*)]. The significant history of these studies has been reviewed by several authors (*4–8*).

Although new publications relating to the Fuchs test have gradually diminished, the possibility of developing a useful cancer test based on Fuchs' findings still appears attractive. Homburger, writing in 1950 (*7*), attempted to relate some of these observations with more recent knowledge of fibrinolysin and antifibrinolysin, and correctly observed that the Fuchs reaction ". . . has held the attention of many investigators for a long time." Thus, some 30 years after its proposal, the value of the Fuchs reaction is not entirely clear.

Two difficulties are reported to arise in the performance of the Fuchs test. The first troublesome point observed by most workers (*4,9*) is

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[2] The authors wish to acknowledge their indebtedness to Dr. George Z. Williams for his interest and help in the preparation of the manuscript.
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

that many carefully prepared specimens of fibrin from cancer patients fail to react. Variations in the reactivity of the fibrin have been ascribed (*9*) to the stage of the disease and to the varying course of the disease in different patients. The second is the difficulty, less frequently mentioned (*3*), of measuring accurately the small increase in nonprotein nitrogen.

Presumably, ways to avoid both of these difficulties could be found and would be worth seeking if confidence in the correctness of Fuchs' observations could be found by confirmation of his earlier work with animals. In the latter instance normal animals were used, and a much greater increase of nonprotein nitrogen was reported. The existence of a species-specific reaction ought, therefore, to be much easier to demonstrate than a disease-specific reaction in cancer patients. Our plan was to proceed to clinical material only after successful confirmation of the animal work.

Methods

We have followed Fuchs in every detail (*1*). Serum and fibrin were prepared as described from sheep and horse blood. Five ml. of serum and 0.1 gm. of dried, pulverized fibrin were placed in dialysis sacks (Visking tubing) and dialyzed against distilled water for 16 hours at 37° C. The vessels used for dialysis were patterned closely after those used by Fuchs and provided an atmosphere of chloroform to retard bacterial growth. After dialysis, aliquots of the outer fluids were analyzed for nitrogen by the usual micro-Kjeldahl technique. The experiment was repeated on several occasions with different samples of fibrin and serum. Where 2 values are shown in table 1, 2 entirely distinct incubations were made. In the controls fibrin was omitted and 0.85 percent NaCl was substituted for serum.

Results

The results of some typical experiments are shown in table 1. For comparison we have included some data from Fuchs.

In our series, dialysis of the serum alone resulted in nitrogen in the outer fluid. This nitrogen is presumably the nonprotein nitrogen of the serum; and, in fact, the values for nonprotein nitrogen (trichloroacetic acid-soluble) and the dialyzable nitrogen agree closely.

On addition of fibrin (of any species) the dialyzable nitrogen is increased. It is, of course, most likely that the additional nitrogen came from the fibrin. Controls to eliminate this factor are not entirely satisfactory because the fibrin does not form a homogeneous suspension in serum but clumps and adheres to the sides of the dialysis sacks in a nonreproducible fashion. We have, however, attempted to deal with the matter to a first approximation by incubating fibrin with 0.85 percent sodium chloride. Table 1 shows that fibrin by itself contains a substantial and variable (according to the batch) amount of dialyzable nitrogen. Fuchs assumed that the fibrin was free from soluble nitrogen. Following Fuchs, we washed the fibrin extensively until the filtrate of a concentrated

TABLE 1.—*Results of experiments with serum and fibrin**

Expt.	Serum	No fibrin	Serum and sheep fibrin	Increase (*less blank*)	Serum and horse fibrin	Increase
A	Sheep	1. 00	1. 66	+0. 11	1. 80	+0. 32
	Horse	1. 24	1. 57	−0. 22	1. 25	−0. 47
	NaCl	0. 00	0. 55	—	0. 48	—
B	Sheep	1. 50; 1. 42	1. 84; 1. 64	−0. 27†	1. 88; 1. 92	+0. 01†
	NaCl	0. 00	0. 55	—	0. 42; 0. 44	—
C	Horse	1. 47; 1. 52	1. 38; 1. 51	−0. 09†	2. 01; 1. 91	+0. 04†
	NaCl	0. 00	0. 04; 0. 03		0. 42; 0. 44	
Fuchs (*1*)	Horse		4. 620‡		0. 0216‡	

*Five ml. serum + 0.1 gm. fibrin incubated in dialysis sacks for 16 hrs. at 37° C. against 35 ml. distilled H_2O. Blank is 0.85 percent NaCl in place of serum. Figures are total N (mg.) in outer fluid.

†Average.

‡Not corrected for fibrin N.

suspension gave no biuret reaction. We have always found, however, that when such material is dried and pulverized, it liberates additional nitrogen to solution. The liberation of nitrogen continues as long as the washing-drying process is repeated. We have, therefore, always used a sample of fibrin as a separate control.

If the increase in nitrogen found on incubation of fibrin and serum is diminished by the value found for fibrin alone, the "corrected" differences are those shown in the table. Of these 8 "corrected" differences, 4 are slight increases while 4 are moderate decreases. The decreases, according to Fuchs' hypothesis, are meaningless; but, if the results are considered to arise from random variability, we find an average decrease of 0.07 mg. N with a standard deviation of ±0.25 mg. N, not significantly different from no change at all.

Discussion

Inhomogeneity of the fibrin and the inability to reproduce exactly the same conditions in the control and experimental vessels are probably the chief factors which lead to variable results. To these must be added the probable failure to achieve equilibrium by the process of stationary dialysis. We did not, however, feel at liberty to depart from the prescribed methods, lest we thereby alter in some way the properties of the system.

Fuchs did not report values for the dialyzable nitrogen in the horse serum; he did, however, accept the value found on incubation with horse fibrin as representing the nonprotein nitrogen of the serum used. He makes no comment on the fact that this value is only about 1 percent of the usual normal values (*10*).

Fuchs, using horse serum, found a large difference in the reactivity of horse and sheep fibrin. Use of the latter gave more than 200 times as much nitrogen in the outer fluid. We could find *no evidence* of *any*

such difference. Further, because Fuchs did not have a control on the fibrins used, we cannot be sure how much, if any, of this increase was caused by proteolysis by a serum enzyme.

We believe that Fuchs' own data fail to substantiate his conclusions. For example, Fuchs gives the result of incubating 100 ml. horse serum and 0.2 gm. sheep fibrin containing 35 mg. N under sterile conditions for 96 hours at 37° as follows: The total soluble (*i.e.*, not removed by simple filtration) N at the start was 13.4680 (*sic*) mg. per ml. and 16.4580 mg. per ml. at the end. The difference, 2.9900 mg. per ml., is erroneously accepted as being the *total* increase in soluble N in the reaction sample (100 ml.). The correct figure is 100 × 2.9900 mg., or 299 mg. Because, at most, only 35 mg. N (the entire N content of the added fibrin) was available, the discrepancy is serious (nearly 850%). Any conclusions that may be drawn from data of this kind are not warranted.

Experiments by Van Der Scheer (*11*) were very similar to our own. He reported small, nonspecific increases in nonprotein nitrogen on incubating fibrin and serum and concluded that species-specific proteolysis did not occur. He assumed, with Fuchs, that the fibrin had no soluble nitrogen and did not include controls.

In our opinion, there are no data to show that proteolysis of any sort regularly occurs under the conditions described by Fuchs. The completely negative nature of these findings is not consistent with Fuchs' postulates and casts doubt on his subsequent work based, in part, on these principles. We have thought, for this reason, that further work in this field would not be rewarding.

Summary

1) The findings of Fuchs regarding species-specific proteolysis of fibrin could not be confirmed.

2) Because the theoretical basis of the Fuchs test for cancer is false, it is doubtful that further work on this test will be rewarding.

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Analysis of Plasma Proteins by Turbidimetry: An Unsuccessful Aid in Cancer Diagnosis [1,2]

J. H. Hill, *M.D.*, *and* Samuel W. Greenhouse, *Mathematical Statistician, Department of Pathology and Oncology, University of Kansas Medical School, Kansas City, Kansas,* and *Biometrics Branch, National Institute of Mental Health,*[3] *Bethesda, Maryland*

In 1952, Parfentjev reported the existence of a pattern of blood-protein changes characteristic of cancer (*1,2*). Variations in concentration of the total protein, total globulin, and 2 protein fractions, the so-called "acid fractions" of serum, were analyzed by turbidimetric methods. At the suggestion of Dr. J. E. Dunn, then of the Cancer Control Division of the National Cancer Institute, an evaluation of the cancer diagnostic possibilities of the plasma-protein pattern, as outlined by Dr. Parfentjev, was carried out after a member of this laboratory staff had worked in Dr. Parfentjev's laboratory for 2 weeks.

This report describes the lack of specificity of this method as a means of cancer detection. Parfentjev has since changed the method (*3*), but evaluation of the modified method has not been carried out.

Method

The blood-protein pattern in cancer patients was believed by Parfentjev to be as follows: increased total serum globulin without an increase in total serum protein, no increase in the albumin-globulin ratio and less "acid fraction" protein precipitate at *p*H 6.0 than at *p*H 5.1. Since he suggested that including the fibrinogen concentration might improve the diagnostic value of the blood-protein pattern, this protein was also measured, which required the use of plasma instead of serum. The plasma from heparinized blood was separated within a few hours and the tests were performed the same day that the blood was drawn. Hemolyzed, icteric, or turbid plasmas were rejected. The test procedure was performed at room temperature between 23 and 26° C.

The protein determinations were made according to the method of Parfentjev and Johnson (*2*). A 10 ml. sample of appropriate concentration of ammonium sulfate solution was added dropwise to the plasma sample in a Coleman 19 × 150 mm. round cuvette over a period of 90 seconds. After standing for 30 minutes, the protein precipitate was

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resuspended by agitation and optical-density measurements at a wavelength of 450 mμ were made promptly in a 1 cm. square cuvette, using a Beckman Model B spectrophotometer. For total-protein determinations, 10 ml. of 50 percent ammonium sulfate solution was added to 0.125 ml. of plasma that had been diluted by adding 1 part plasma to 2 parts 0.85 percent sodium chloride solution. Total-globulin estimations were made in the same manner as for total protein, except that a 27 percent ammonium sulfate solution was used. The albumin concentration was obtained by subtraction. The optical density of a mixture of 10 ml. of a buffer solution added to 0.05 ml. of undiluted plasma indicated the concentration of the *p*H 5.1 acid fraction. The buffer solution was 0.5 ml. of a 2.5 percent solution of sodium acetate diluted to 300 ml. with water. To this solution was added sufficient glacial acetic acid so that the final *p*H of the mixture of plasma and buffer was 5.1. The *p*H 6.0 acid fraction was similarly estimated, using a 5 percent instead of a 2.5 percent sodium acetate solution and adjusting the buffer solution so that the final *p*H, after addition of a 10 ml. aliquot to the plasma, was 6.0. Estimates of fibrinogen were made by turbidimetry (*4*), using 0.5 ml. of plasma to which was added 0.5 ml. of 0.85 percent sodium chloride solution and then 9 ml. of a solution of 12 percent ammonium sulfate. Fibrinogen readings were made 15 minutes after addition of the salt solution.

The optical-density values of turbidity were translated into gm. percent for total protein, total globulin, and fibrinogen—the first 2 by utilizing a conversion factor and the last by use of a line graph. The total-protein factor was obtained by making simultaneous biuret and turbidity total-protein determinations on 73 different samples of plasma. The mean biuret total-protein concentration was 6.5 gm. percent and the mean turbidity value was 0.421 optical-density units, giving a factor of 15.5. The globulin factor was obtained by making micro-Kjeldahl and turbidimetric protein determinations on a sample of commercial human γ-globulin. The factor for translating total-globulin optical-density values to gm. percent was found to be 13.6. Fibrinogen concentration in gm. percent was read from a line graph derived by plotting the fibrinogen turbidity optical-density values of a group of 23 plasmas and their fibrinogen concentrations were determined by the method of Morrison (*5*). The concentration of the acid fractions was expressed in terms of the optical-density reading.

Clinical Material

Blood-protein patterns were studied in a group of 146 patients in the University of Kansas Medical Center. Of these, 54 had cancer, 64 had a noncancerous disease, and 28 were considered to be normal. The mean age of the 29 cancerous men was 58 years (range 78–21) and of the 25 cancerous women, 54 years (range 81–24). In the group of 64 noncancerous individuals were 33 men whose mean age was 51 years (range 82–15) and 31 women whose mean age was 44 years (range 77–16). There were 16 men and 12 women in the normal group with mean ages of 47 years

(range 76–16) and 31 years (range 66–17), respectively. The sites of the cancerous diseases were: cervix uteri, 11; prostate, 7; breast, 6; malignant lymphoma, 6; gastrointestinal tract, 4; tongue, 3; lung, 3; thyroid, 2; sarcoma of soft tissue, 3; primary site undetermined, 3; and miscellaneous sites, 6. The noncancerous-disease group was made up of 37 with types of inflammation, 14 with types of hyperplasia, 11 with cardiovascular disease, and 2 with miscellaneous diseases. The results of tests on 17 patients were eliminated from this study owing to uncertain diagnosis. Final classification of the patients was made after the test was performed. All cancer diagnoses were based on tissue examination.

Evaluation of the Micro Method of Total Plasma-Protein Determination

A preliminary study was made of the technical errors to be avoided, the accuracy of the method of protein measurement, and the variations in results to be expected.

The rate of addition of the ammonium sulfate solution, changes in turbidity occurring on standing, effect of temperature, and effect of minor variations in the hydrogen-ion concentration of the ammonium sulfate solutions were investigated as possible sources of error. Rapid addition of salt solution to the plasma led to poor reproducibility of the turbidity, but when 10 ml. of salt solution was added dropwise over a period of 90 seconds the reproducibility was satisfactory. Significant change in turbidity of the fibrinogen and *p*H 6.0 acid-fraction mixtures of plasma and ammonium sulfate occurred on standing. The fibrinogen turbidity increased rapidly during the first 10 minutes, with little subsequent change. Often, after 20 minutes' time, coarse flocculent clumps were formed which did not disperse upon reagitation of the mixture. There was a moderate, gradual increase in turbidity of the *p*H 6.0 acid-fraction mixture over a period of 20 minutes. Therefore, all turbidity measurements were made after allowing the plasma and ammonium sulfate mixtures to stand for 30 minutes, except those for the fibrinogen, which were made after 15 minutes. Temperature variations occuring in the laboratory and minor changes in *p*H of the ammonium sulfate reagent were not critical.

The accuracy of the turbidity technique was estimated relative to a biuret and to a micro-Kjeldahl method of protein determination. The mean total-protein concentration in 72 plasmas, determined simultaneously by a biuret method (*6*) and by the turbidity technique, was found to be 7.3 and 7.2 gm. percent, the standard deviation 0.61 and 0.67 gm., and the coefficient of variation 8.4 and 9.4 percent, respectively. The difference in mean protein concentrations by these 2 methods was not statistically significant. The turbidity method, the biuret procedure, and the micro-Kjeldahl method were used to determine the total protein of a group of 20 plasmas without statistically significant differences. It was concluded that the turbidity technique was a reasonably accurate method of protein estimation.

Reproducibility of Protein Measurements

In order to determine the reproducibility of various protein measurements, 5 samples of blood were obtained from each of 6 plasma pools. Three independent determinations were made on each sample, giving a total of 15 determinations per pool. From this experimental situation, it was possible to estimate 2 basic sources of variation: 1) the variation among the 3 determinations per sample, which we denote as measurement error, and 2) the variation among the 5 samples per pool, which can be denoted as sampling error.

Table 1 presents a summary of all the information. Line 1 presents the range of mean values of the various components measured in the 6 plasma pools. Lines 2 and 3 relate to measurement error. Line 2 gives the range of measurement error over the 6 pools (a standard deviation of measurement obtained for each sample and pooled over the 5 samples per pool). Line 3 presents the mean coefficient of variation of measurement error of all 6 pools and the range of the 6 coefficients of variation. This was obtained in each case as 100 times the ratio of the standard deviation of measurement to the mean value. It will be seen that the average coefficient of variation of measurement error ranged from a low of 2.2 percent for *p*H 6.0 acid fraction to 6.1 percent for total globulin.

Lines 4, 5, 6, and 7 relate to the second source of variation mentioned, namely, sampling error. The variation observed among 5 samples with 3 observations each obviously includes measurement error. By applying the technique of components of variance, estimates were made of the variability of true sample values, excluding any error due to measurement. These are presented in lines 4 and 5. Line 4 shows the estimated standard deviation of true sample values and line 5 presents the estimated average coefficient of variation of true sample values together with the range of such coefficients over the 6 pools. According to our estimates, the variation of true values among samples taken from the same pool is on the average of the same magnitude as measurement error.

Lines 6 and 7 refer to measures of variability between samples, including measurement error. If one were to take many samples and make 1 observation per sample, the expected standard deviations would be as given in line 6 and the expected coefficients of variation for pools having about the same mean values as observed here, as presented in line 7. It will be seen that fibrinogen and globulin, having 10.2 and 8.4 percent coefficients of variation, respectively, varied more than the other 3 fractions, whose coefficients of variation were less than 5 percent.

It was mentioned that the estimate of variation between true sample values drawn from the same pool was of the same magnitude as measurement error. This fact points to the basic random variation inherent in sampling, namely, that samples of plasma tend to have protein contents that differ among themselves and also differ from that existing in the pool. All other sources of variation, such as measurement error, must then be added to this basic variation. On the other hand, if this basic sampling variation is thought to be too large, one can attribute this to some possible

TABLE 1.—*Variation in protein measurements**†

	Total protein	Globulin	Fibrinogen	Acid fraction	
				*p*H 5.1	*p*H 6.0
1. Range of protein concentration of 6 pools	6. 1–8. 1‡	1. 8–2. 8‡	0. 27–0. 30‡	0. 194–0. 446	0. 256–0. 430
2. Range of standard deviation of measurement error in 6 pools	0. 19–0. 29	0. 11–0. 20	0. 0032–0. 0054	0. 0024–0. 175	0. 0050–0. 0097
3. Average coefficient of variation of *measurement error* in 6 pools (*percent*)	*3. 4* (2. 3–4. 8)	*6. 1* (4. 2–8. 5)	*5. 6* (3. 6–9. 5)	*3. 4* (0. 9–5. 1)	*2. 2* (1. 8–2. 8)
4. Range of standard deviation of estimated true sample values in 6 pools	0. 22–0. 37	0. 09–0. 27	0. 0022–0. 0094	0. 0026–0. 0146	0. 0033–0. 0126
5. Average coefficient of variation of the *estimated true sample values* in 6 pools (*percent*)	*3. 2* (3. 0–4. 7)	*5. 7* (3. 3–10. 5)	*9. 2* (3. 7–10. 9)	*2. 5* (0. 8–5. 5)	*2. 3* (1. 3–2. 9)
6. Range of standard deviation of sample values, *including measurement error*	0. 33–0. 43	0. 20–0. 29	0. 0039–0. 0099	0. 0045–0. 0182	0. 0060–0. 0148
7. Average coefficient of variation of *sample values* of 6 pools *including measurement error* (*percent*)	*4. 7* (4. 6–6. 4)	*8. 4* (7. 3–11. 2)	*10. 2* (6. 6–13. 0)	*4. 2* (1. 7–5. 9)	*3. 2* (2. 3–3. 8)

*Tabulation of the variation in protein measurements when 5 samples, each tested in triplicate, were taken for analysis from a pool of plasma. The 6 pools analyzed in this manner had the protein content indicated in line 1. Shown are the standard deviation (S.D.) and coefficient of variation (C.V.) of the measurement error (lines 2 and 3), of the estimated true sample values (lines 4 and 5), and of the samples based on 1 observation per sample, including the measurement error (lines 6 and 7).

†Figures in parentheses are the range of values.

‡Grams percent.

nonrandom element, such as inadequate stirring of the pool between removal of successive samples.

The measurements of fibrinogen concentration were computed from the least squares line $Y = 0.255 + 0.425\ X$ where Y is concentration of fibrinogen and X is the optical density. This line estimates the mean concentration of fibrinogen expected at a given X value. The reliability of this estimate is a function of the variation of the points about the line and the distance of the given X from the mean X. The ratio of this standard error of a computed mean value to the computed value itself is given in table 2 as a coefficient of variation for 5 values of X within the range of observations of X's. The standard-deviation values for fibrinogen given in table 1 were calculated from the optical-density values.

TABLE 2.—*Coefficients of variation of fibrinogen measurements for selected optical-density values*

Optical density	Computed* average fibrinogen concentration (*gm. percent*)	Standard error of average fibrinogen concentration (*gm. percent*)	Coefficient of variation (*percent*)
0. 1	0. 298	0. 0235	8
0. 3	0. 383	0. 0170	4
0. 5	0. 468	0. 0237	5
0. 7	0. 553	0. 0371	7
0. 9	0. 638	0. 0521	8

*Computed from $Y = 0.255 + 0.425\ X$.

The day-to-day variation in the plasma proteins of 5 individuals over a period of 5 days was measured by calculating the standard deviation and coefficient of variation of the daily protein measurements. The day-to-day variation was considerably greater than that due to sampling or measurement error. When the standard deviations were based upon assuming 1 sample per individual and 1 observation per sample, the coefficients of variation were: total protein 10.8, globulin 17.1, fibrinogen 26.3, *p*H 5.1 acid fraction 34.1, and *p*H 6.0 acid fraction 18.6 percent. A comparison was made of the variations in samples taken at the same time and samples taken at different times by comparing the observed variances of the samples. For total protein they were the same, for globulin the latter was about 4½ times greater, for fibrinogen about 8 times greater, for *p*H 5.1 acid fraction they were nearly the same, and for *p*H 6.0 acid fraction the variance was 28 times greater for samples taken at different times than for samples taken at the same time.

Results

The cancer selectivity was determined for various combinations of total protein, for globulin, and for the albumin-globulin ratio. The 6 combinations selected for analysis were those shown in table 3. Patients who had lowered total-protein and elevated globulin concentrations with a lowered

albumin-globulin ratio were classified as cancerous. The arbitrary range of values for cancer was: total protein, less than 7.5 gm. percent; albumin-globulin ratio, less than 1.5; and various globulin concentrations 2.5 to 3.9 gm. percent. Of these combinations, listed in table 3, "B" was closest to Parfentjev's specifications.

TABLE 3.—*Cancer diagnostic accuracy of plasma-protein determinations by the turbidity technique**

Combination	Normals 28	Cancers 54	Other diseases 64
A. Total protein less than 7.5 A/G ratio less than 1.5 globulin between 2.5 and 3.9	8 *29*	30 *56*	20 *31*
B. Total protein less than 7.5 A/G ratio less than 1.5 globulin between 3.0 and 3.9	1 *4*	18 *33*	10 *16*
C. A/G ratio less than 1.5 globulin between 2.5 and 3.9	12 *43*	35 *65*	20 *31*
D. Total protein less than 7.5 globulin between 2.5 and 3.9	9 *32*	34 *63*	27 *42*
E. Total protein less than 7.5 globulin between 3.0 and 3.9	1 *4*	18 *33*	11 *17*
F. A/G ratio less than 1.5 total protein less than 7.5	8 *29*	33 *61*	25 *39*

*Figures in italics are percentages.

The cancer selectivity of these combinations shown in table 3 ranged from 33 to 65 percent correct positive results, but false positive results in the normal group ranged from 4 to 43 percent and in the group of non-cancerous but ill individuals it was from 16 to 42 percent. When the false positive results were acceptably low in the normal group, *i.e.*, 4 percent with combination "B" or "E," the correct positive results were only 33 percent and the false positive results in the ill patient group were 16 and 17 percent. The 2 combinations having the best cancer selectivity, *i.e.*, "C" and "F," with 65 and 61 percent correct positive results, respectively, also had the highest false positive results in the normal group, *i.e.*, 43 and 29 percent.

In view of the poor cancer selectivity of these combinations, inclusion of fibrinogen and the acid fractions in the panel of plasma-protein measurements was not considered justified.

Discussion

An investigation of cancer diagnostic procedures based upon empirical changes in the blood proteins may be questioned, since very few tumors have been associated with a specific type of blood-protein abnormality.

Mider and co-workers (*7*) found an abnormal distribution of the plasma proteins, as determined by electrophoresis in 222 individual cancer patients. The abnormalities became more pronounced as the cancerous growth increased in extent and consisted of decreased albumin concentration and increased total globulins, usually due to increase in fibrinogen and α-globulin, whereas the changes in β- and γ-globulin concentration were much less. Although these changes were statistically valid, they were not precise enough to form the basis of a diagnostic test. Bernfeld and Homburger (*8*) found decreased albumin and increased α-globulin concentrations in the blood of mice bearing sarcoma 180. Reference was made by Loeper (*9*) to a paradoxical hyperproteinemia in the blood of a few cancer patients in which the serum globulin was greatly increased. This was thought to be due to liberation into the blood of proteins from large tumors undergoing degeneration. The extreme changes described by this author were not encountered in the present study.

A procedure similar in some respects to Parfentjev's "acid-fraction" analysis was described by Greenspan (*10*). It was based on the turbidity of serum diluted 1:60 with 0.01 ionic strength acetate buffer at *p*H 4.42. Increased concentration of these "acid-precipitable globulins" occurred in two thirds of the patients tested but the increase was apparently nonspecific.

When Parfentjev and Johnson (*2*) reported on the results of their analysis of about 2,000 samples of blood, the blood-protein pattern had been extended to include fibrinogen, pseudoglobulin, and γ-globulin; the *p*H 6.0 acid fraction was not mentioned. The protein fractions were now considered with reference to the total protein concentration. With these revised criteria, they believed sick and healthy individuals could be differentiated with an accuracy of over 90 percent. With the abnormal blood-protein patterns they could detect approximately 80 percent of the patients with malignancy. These revised criteria did not appear to offer real promise of greater discrimination between cancerous and noncancerous individuals, and further work along these lines was not carried out in this laboratory.

The turbidity technique of measuring protein concentration depends upon the complex reactions involved in salt precipitation of proteins. The reproducibility of these reactions relies upon the critical control of a variety of factors, including rate of mixing, concentration of reactants, temperature, and time elapsed after mixing. The turbidity observed in the test is, after all, governed by the particle-size distribution of precipitated protein. These considerations emphasize the problems that may arise in determining protein concentration by the turbidity technique.

Summary and Conclusions

No cancer-specific plasma-protein pattern could be demonstrated when blood from 54 cancer patients, 64 with noncancerous disease, and 28 normal persons were compared. The plasma proteins were determined by a turbidimetric micro method.

The concentration of the following plasma proteins formed the basis of the comparison: total plasma protein, total globulin, and the albumin-globulin ratio. Measurements were made on fibrinogen and the *p*H 5.1 and *p*H 6.0 "acid-fractions" but their inclusion in the panel of plasma proteins selected for statistical analysis was not justified in view of the poor cancer selectivity of the panel.

These results conform to those of other similar studies in which the protein fractions were determined by electrophoresis. It was concluded that the existence of a cancer-specific blood-protein pattern remains to be demonstrated.

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An Evaluation of the Zuccala Cancer Test [1,2]

J. H. Hill, *M.D.*, *Department of Pathology and Oncology, University of Kansas Medical School, Kansas City, Kansas*

The Zuccala cancer test (*1,2*) was based on the degree of hemolysis produced by a patient's serum on sheep erythrocytes that had been "antigenized" by exposure to an "antigen" of Zuccala's manufacture. Serums from cancerous, tuberculous, and pregnant individuals were said to induce hemolysis in higher dilution than serums from other patients. The evaluation of this test was carried out at the request of the Committee on Cancer Diagnosis and Therapy of the National Research Council, which supplied the 3 lots of antigen and the test procedure.

Three different antigens were employed in testing serums of 75 cancerous and 233 noncancerous patients. Since omission of the antigen made no appreciable difference in the degree of hemolysis, it was concluded that the antigen was probably inert. However, it was discovered that more hemolysis occurred with serums from cancerous patients, so the results were analyzed for cancer selectivity, even though the antigen appeared to contribute nothing to the results of the test.

Material and Methods

A group of 308 patients in the University of Kansas Medical Center, 75 having cancerous and 233 noncancerous diseases, was tested. The extent of the cancerous growth and the types of noncancerous diseases were as outlined in part A of table 1. The primary sites of the cancerous growths were as indicated in table 2. The average age of the 30 women and 45 men in the cancerous group was 64 years. The average age of the 142 women and 91 men in the noncancerous group, except the patients in pregnancy, was 54 years. All serums were tested as unknowns within 24 hours after withdrawal of blood. Final classification of the patients was made later on the basis of the completed clinical record. Cases in which the clinical diagnosis was uncertain were omitted. All cancer diagnoses were made on the basis of tissue examination.

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[2] This investigation was supported in part by research grant CS-9051 from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service, and an institutional grant from the American Cancer Society.

TABLE 1.

A. Distribution of patients with and without cancer		B. Incidence of 3 or 4 plus reaction in 1:10 serum-dilution tube, according to lot of antigen used in the test							
Disease	No. of patients	Lot I		Lot II		Lot III		Total	
Cancerous patients		+	−	+	−	+	−	+	−
Untreated	*55*	*9*	*13*	*12*	*5*	*12*	*4*	*33*	*22*
(Localized)	(14)	(3)	(1)	(4)	(1)	(4)	(1)	(11)	(3)
(Regional spread)	(19)	(1)	(4)	(5)	(3)	(5)	(1)	(11	(8)
(Distant spread)	(22)	(5)	(8)	(3)	(1)	(3)	(2)	(11)	(11)
Treated	*20*	*4*	*6*	*0*	*1*	*9*	*0*	*13*	*7*
(Localized)	(8)	(1)	(6)	(0)	(0)	(1)	(0)	(2)	(6)
(Regional spread)	(11)	(2)	(0)	(0)	(1)	(8)	(0)	(10)	(1)
(Distant spread)	(1)	(1)	(0)	(0)	(0)	(0)	(0)	(1)	(0)
TOTAL	75	13	19	12	6	21	4	46	29
PERCENT	—	**40**	—	**66**	—	**84**	—	**62**	—
Noncancerous patients									
Minimal or no disease	48	3	13	1	16	13	2	17	31
Postoperative or inflammation	56	3	10	3	25	13	2	19	37
Arteriosclerosis	30	0	5	0	6	14	5	14	16
Pregnancy	29	8	5	7	7	1	1	16	13
Hyperplasia	28	1	9	2	8	6	2	9	19
Benign tumor	5	0	0	0	1	3	1	3	2
Miscellaneous disease	37	0	9	3	15	8	2	11	26
TOTAL	233	15	51	16	78	58	15	89	144
PERCENT	—	**23**	—	**17**	—	**79**	—	**38**	—

The following test procedure was used: Into each of 5 Kolmer-Wassermann tubes, 0.25 ml. of diluted serum (1:5, 1:10, 1:20, 1:40, and 1:80 dilutions, respectively), 0.5 ml. of antigenized sheep cells, and 0.5 ml. saline were placed and mixed well. After 30 minutes' incubation in a 37° C. water bath the tubes were placed for 2 hours in a refrigerator. The amount of hemolysis in each tube was then read on a 0 to 4+ scale, the tube being centrifuged to facilitate estimation of hemolysis. The hemolytic effect of serum was expressed as the sum of the hemolysis readings in all 5 tubes, graded on a 0 to 4+ scale. Maximum hemolysis would then be indicated by the number 20. The sheep erythrocytes were obtained from the Brown Laboratory of Topeka, Kansas, which tested the cells for their resistance and fragility by use of a titration with Kolmer antigen and anti-sheep hemolysin. Before use, the cells were washed 3 times in 0.85 percent sodium chloride solution; they were then used as a 5 percent suspension. Dilute antigen was made daily from the stock antigen by appropriate dilution with 0.85 percent sodium chloride solution, as directed on the label of the stock-antigen vial. A volume of diluted antigen equal to the required volume of 5 percent sheep-erythrocyte suspension was poured into the erythocyte suspension and mixed by pouring back and forth at least 3 times. After 10 minutes the "antigenized" cells were ready for use. The serums were *not* inactivated.

TABLE 2.—*Incidence of various primary-tumor sites in the group of cancerous patients*

Extent of cancer	Site of primary growth	No. of patients*
A. Untreated		
Localized tumors	Urinary bladder	3
	Cervix uteri	2
	Prostate	3
	Skin	3
	Gastrointestinal tract	2
	Larynx	1
		14
Tumor with spread to regional nodes or widespread local invasion.	Gastrointestinal tract	5
	Lung	4
	Pancreas	2
	Prostate	2
	Cervix uteri	2
	Malignant melanoma, epiglottis, lymphoepithelioma, and sarcoma—1 each	4
		19
Distant metastases	Primary site unknown	5
	Gastrointestinal tract	5
	Prostate	3
	Leukemia and lymphosarcoma	4
	Ovary	2
	Lung, cervix uteri, and breast—1 each	3
		22
B. Treated		
Localized tumors	Breast	2
	Skin	2
	Cervix uteri, prostate, and testis—1 each	3
		8
Tumors with spread to regional nodes or widespread local invasion.	Gastrointestinal tract	4
	Cervix uteri	3
	Thyroid, malignant melanoma, gingiva, and skin—1 each	4
		11
Distant metastases	Hodgkin's disease	1
	Primary site undetermined	2
		3

*Figures in italics are totals for each group.

Results

Duplicate tests were performed simultaneously on 99 serums; the duplicates differed only in omission of the antigen from 1 of the pair. The results of testing the 3 antigens in this manner, shown in table 3, indicated that the antigens were inert under the conditions employed, since the same degree of hemolysis occurred regardless of the presence or absence of antigen. Serums from the 10 untreated and the 6 treated cancer patients in the group gave results no different from those of the entire group. The effect of adding 0.1 ml. of 1:30 guinea-pig complement to 1

TABLE 3.—*Effect of omitting antigen upon hemolysis in the Zuccala test**

Antigen	No. of patients	Average hemolysis per test	
		With Zuccala antigen	Without Zuccala antigen
I	10	7. 2	7. 1
II	45	5. 0	5. 1
III	44	8. 9	9. 7

*Hemolysis was graded on a 0 to 20 scale.

set of duplicate tubes was measured, because Zuccala had suggested that complement be added to the reactants. In tests on 76 serums the average hemolysis per test without added complement was 8.3 and with added complement, 10.0. This difference of 1.7 in the average hemolysis per test was thought to be highly significant, since the probability was about 1 in 500 that the difference was due to chance ($P = 0.0018$). A similar comparison was made of the effect of adding 0.1 ml. of a 1:20 complement dilution to 18 serums. The average hemolysis per test was 11.3 with the 1:20 complement dilution and 9.1 without complement. Statistical analysis indicated that the observed average increase in hemolysis of 2.2 by addition of 0.1 ml. of 1:20 complement dilution was probably not due to chance ($P = 0.08$). It was concluded that 0.1 ml. of 1:20 or 1:30 complement dilution enhanced the hemolysis observed in the test.

Although the antigen was inert, more hemolysis occurred with cancerous serum; therefore, the results were analyzed to determine cancer selectivity. The average hemolysis per test was 7.4 in the cancerous group (75 serums) and 6.9 in the noncancerous group (233 serums). The difference in these averages was small and was probably due to chance ($P = 0.46$). Inspection of the results suggested that the hemolysis occurring in the second tube in the test, *i.e.*, the tube containing a 1:10 serum dilution, might afford the best cancer selection. Accordingly, a 3 or 4+ hemolysis reading in this tube was arbitrarily considered indicative of cancer. The results of this analysis, shown in part B of table 1, indicated that more hemolysis occurred with cancerous serum when lots I and II of the antigen were being tested, *i.e.*, 40 and 66 percent correct positive and 23 and 17 percent false positive, respectively. When lot III was being tested the correct and false positives were almost the same, *i.e.*, 84 and 79 percent, respectively. The over-all cancer selectivity was 62 percent correct and 38 percent false positive results.

No better cancer selectivity was found in the small group of serums tested with the addition of guinea-pig complement, and further testing with it was not believed to be warranted.

Discussion

It is regrettable that the author of this test neglected to include suitable controls in his test procedure. Otherwise he could have learned sooner

about inertness of the antigen. The test probably measured the amount of anti-sheep hemolysin in patients' serums, a property of blood which has not been considered to be correlated with any one human disease. The concept that the heterophile hemolysin concentration of serum is helpful in the management of human disease is, of course, empirical. However, it must be conceded that empirical heterophile serum reactions might be shown to have a place in the diagnosis of cancer. Another heterophile serum reaction, *i.e.*, measurement of the anti-sheep hemagglutinins in patients' serum, has become a valuable aid in the diagnosis of infectious mononucleosis. In the absence of specific bases for cancer tests, empirical methods require evaluation if they are claimed to be cancer-selective.

The cancer selectivity of the hemolysin reaction found in testing the first 2 lots of antigen was not much better than that of other proposed cancer tests (*3*). The complete lack of cancer selectivity of the hemolysin reaction in tests of the third lot of antigen (shown to be inert) was somewhat disturbing and was attributed to possible variation in sensitivity of the sheep erythrocytes. Although the supplier tested each batch of sheep erythrocytes for their sensitivity to the anti-sheep hemolysin used in the Kolmer test for syphilis, 1 of the lots of cells was completely resistant to the hemolysin in our patients' serums. These same serums had average hemolytic activity when tested with sheep cells from other sources. Variation in sensitivity of the sheep erythrocytes could not be estimated but would not be a serious obstacle to a useful test if large differences in hemolysis were being measured. The distinction between 2 and 3+ hemolysis was necessarily arbitrary in reading some of the tests.

The selective absorption by guinea-pig kidney of the heterophile antibody in the serum of patients with infectious mononucleosis suggests the existence of other heterophile antibodies that might be cancer-selective. Using serum absorbed with homogenates of various tumors might reveal hitherto unsuspected antibodies. Future investigation along these lines could be carried out with profit.

Summary and Conclusion

The Zuccala cancer test was moderately cancer-selective during one part of this evaluation and nonselective during another period of the study. The test measured the anti-sheep hemolysin activity of serum, which was apparently not related to a particular human disease. The antigen employed in the test was inert.

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Serum Lactic Dehydrogenase in Cancer Patients [1,2]

J. H. HILL, *M.D., Department of Pathology and Oncology, University of Kansas Medical School, Kansas City, Kansas*

Significantly increased serum lactic dehydrogenase (SLD) activity in cancerous patients and in some pregnant women was reported by Hill and Levi (*1*) after testing 43 normals, 10 pregnant women, 42 tuberculous patients, 51 patients with neoplastic disease, 3 with benign tumors, and 3 with cardiovascular diseases. Subsequently, Bierman *et al.* (*2*) found elevated SLD activity in 121 of 132 cancerous patients. Of 94 patients with non-neoplastic diseases, only 3 had elevated SLD activity. All 64 healthy individuals had normal SLD levels and all of 50 leukemic patients, either untreated or in relapse, had elevated levels.

The cancer selectivity described by those authors warranted further evaluation of SLD activity as a cancer-selective procedure. Although the group of cancerous patients in the present study had an average SLD activity well above normal, so did the group of patients with noncancerous disease. The reaction was not found to be cancer-selective.

Method

SLD activity was determined by the decrease in optical density of a solution of the reactants read at a wavelength of 340 mμ during a period of 30 minutes. The solution contained sodium pyruvate, reduced diphosphopyridinenucleotide (DPNH), phosphate buffer, sodium bicarbonate, and serum. Lactic dehydrogenase catalyzes the reduction of pyruvate to lactate by DPNH; the oxidation of DPNH to DPN is reflected in decreased optical-density measurements at 340 mμ.

The procedure described by Hill and Levi (*1*) was followed, with these exceptions: It was found that the DPNH solution made up in the *p*H 7.8 buffer could be kept in the refrigerator for as long as 5 days. The Beckman Model B spectrophotometer was found not to be well-adapted for this type of analysis and a Beckman Model DU spectrophotometer was used instead, with temperature maintained at 37.5° C. by circulating water through Beckman thermospacers. The reactants were mixed by

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inverting the cuvette 5 or 6 times instead of stirring. All serums were tested within 6 hours after the blood was drawn. A few serums that could not be tested immediately were preserved by freezing at −20° C. for not longer than 7 days, a step which did not impair SLD activity.

Icteric or turbid serums and those with any evidence of hemolysis were rejected. The diagnoses were not known at the time of the test. All cancer diagnoses were based on tissue examinations, and patients with uncertain diagnoses were excluded. Classification of each patient was made after a careful evaluation of his completed hospital record.

Serums from 56 normal individuals and 87 cancerous and 309 noncancerous patients in the University of Kansas Medical Center were tested for their SLD activity. The normal group consisted of 41 blood donors and 15 office workers at the Medical Center. Of these 40 were men (average age 44 years) and 16 were women (average age 41 years). In the cancer group were 40 men (average age 62 years) and 46 women (average age 61 years). There were 130 men (average age 56 years) and 179 women (average age 44 years) in the noncancerous group. The types of noncancerous diseases were distributed as shown in table 1. In the cancer group were 60 untreated patients; 22 had localized tumors (gastrointestinal tract, 6; prostate, 6; cervix uteri, 2; basal-cell carcinoma, 2; breast, 2; and endometrium, maxilla, bladder, and larynx, 1 each), 11 had tumors that had spread to regional nodes or showed widespread local invasion (vagina, cervix uteri, ovary, colon, stomach, esophagus, lung, nasopharynx, lymphoepithelioma, breast, and pancreas, 1 each); 27 had distant metastases (leukemia, 6; gastrointestinal tract, 6; primary site undetermined, 4; cervix uteri, 4; lung, 3; malignant lymphoma, lymphoepithelioma, ovary, and kidney, 1 each). There were 27 treated cancerous patients, of whom 16 had localized tumors (gastrointestinal tract, 5; cervix uteri, 3; skin, 2; malignant lymphoma, 2; kidney, bladder, endometrium, and breast, 1 each), 7 had regional spread to lymph nodes or adjacent structures (gastrointestinal, 3; sarcoma, 2; tongue and gingiva, 1 each), and 4 had distant metastases (prostate, 2; breast and cervix uteri, 1 each).

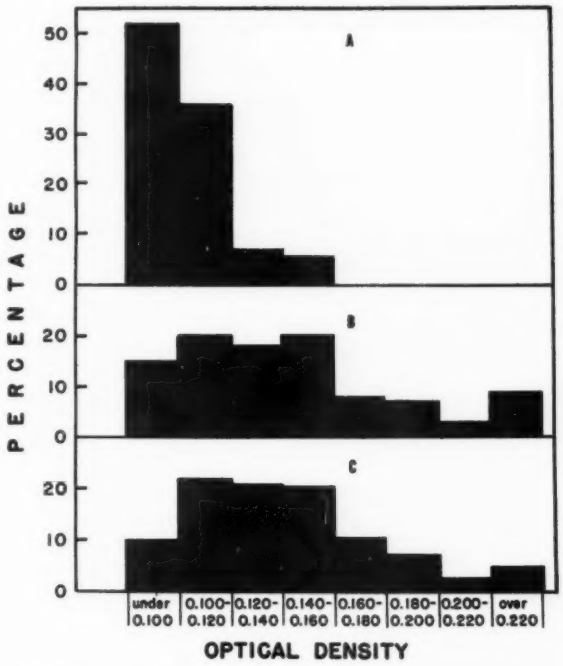
Reproducibility of Results

Variation in results in replicate testing on a single day was not great. When 3 different serums were tested 8, 6, and 6 times in a single day, the means, standard deviations, and coefficients of variation of the optical-density measurements were: 0.152, 0.0014, and 0.9 percent; 0.078, 0.0037, and 4.7 percent; 0.049, 0.004, and 8.8 percent. As expected, results in the lower range of values varied more than those in the higher ranges; *i.e.*, the coefficient of variation was 8.8 percent when the mean result was 0.049, compared with 0.9 percent when the mean result was 0.152. When the same serum was tested on different days, variation in results was not much greater than when the same serum was repeatedly tested on the same day. Thus, when a serum was divided into 14 tubes, which were kept frozen at −20° C., and a different tube was tested on each successive day, a mean result of 0.093 was obtained, and the standard deviation was 0.0065,

while the coefficient of variation was 6.9 percent. Approximately 2 to 4 times more variation in results occurred when different serums of the same patient were tested daily for 5 days. Serums from 3 patients tested in this manner had means, standard deviations, and coefficients of variation, respectively, as follows: 0.086, 0.014, and 16.3 percent; 0.091, 0.0087, and 9.6 percent; 0.089, 0.017, and 19.2 percent. The day-to-day variation in results might reflect changes in the serum constituents or different amounts of contamination with LD-rich breakdown products of the blood cells, released incident to separation of the serum. It was concluded that variation in results due to vagaries in technique was not excessive but that considerable variation in results could be expected from testing different specimens from the same patient. These findings correspond, in general, to the experience with replicate *versus* repetitive testing of other biochemical properties of blood.

Results

Significantly lower SLD activity was present in the normal group (*A*) than in the cancerous (*B*) and noncancerous groups (*C*) (text-fig. 1). In the latter 2 groups the SLD levels were essentially the same. The mean SLD activity and range of values in the various categories of patient



TEXT-FIGURE 1.—Percentage of patients in each group, according to serum lactic dehydrogenase activity expressed in optical density. *Group A* consisted of 56 normal persons, *group B*, 87 cancerous patients, and *group C*, 309 noncancerous patients.

groups were as shown in table 1. The average SLD activity in the normal group (0.098) was significantly less than the average SLD activity in the cancerous and noncancerous groups (0.149 and 0.143, respectively). The distribution of results in each category, according to range of optical-density values, was as shown in table 2. None of the 56 individuals in the normal group had SLD values over 0.200, while 12.6 percent (11 of 87) of the cancerous group and 7.7 percent (24 of 309) of the noncancerous group had SLD values over 0.200. The highest incidence (27%) of elevated SLD in the cancerous group was in the group of 11 patients with carcinoma of the uterine cervix. In the cardiovascular-disease group 17.5 percent had SLD values of 0.200 or over, while 7 percent or less of the other types of noncancerous disease were in this range. SLD activity appeared to be as selective for patients with cardiovascular disease as it was for patients with cancer.

Of the 6 patients whose SLD was over 0.300, 3 had heart disease—2 had fresh myocardial infarcts, 1 and 3 days old, and the third died 3 weeks later of the effects of severe arteriosclerotic cardiorenal disease. One of the other 3 patients had a bleeding duodenal ulcer but did not receive transfusions and another was a woman 1 day *post partum*; the third had a duodenal polyp and required a transfusion for pernicious anemia.

Discussion

An explanation for this failure to confirm the results reported by Hill and Levi (*1*) and by Bierman and co-workers (*2*) is not readily apparent. In the former report all normals were under 0.100, with a median SLD activity of about 0.085 estimated from the published chart. The normal group of our study had a median SLD value of 0.090 and a maximum of 0.143. The median SLD value in Hill and Levi's cancerous group was about 0.210 compared to 0.130 in this study. Since these authors did not describe the types of neoplastic disease in their cancerous group, one cannot be certain that the 2 studies are similar in this respect. The median SLD of their 10-patient pregnancy group was about 0.135 compared to 0.140 in our 40-patient pregnancy group. Their 42-patient tuberculosis group had no counterpart in this clinical material. The other 27 miscellaneous patients that they studied all had SLD values under 0.100, in contrast to the higher values found in this study. Optimal conditions for demonstration of SLD have recently been reported by Hill (*3*) at a *p*H of 7.2 instead of 7.8. In summary, these 2 studies seem to agree in the results of testing normal and pregnancy groups but not in those for the cancerous and noncancerous-disease groups.

After completion of this work, a report on lactic dehydrogenase activity in blood was published by Wroblewski and LaDue (*4*), whose method was similar to the one employed by us. Translating their results into terms of optical density revealed that their normal mean value was 0.141, compared with 0.098 in our study, and their range was 0.078 to 0.255 compared to our range of 0.043 to 0.143. While they were primarily concerned with the changes in SLD incident to myocardial infarction,

TABLE 1.

A. Distribution of patients with and without cancer		B. Serum lactic dehydrogenase activity*	
Group	No. of patients	Mean	Range
Cancerous			
Untreated	*60*	*0. 150*	*0. 060–0. 344*
(Localized)	(22)	(0. 130)	0. 073–0. 164
(Regional spread)	(11)	(0. 114)	0. 060–0. 179
(Distant spread)	(27)	(0. 181)	0. 088–0. 344
Treated	*27*	*0. 149*	*0. 066–0. 583*
(Localized)	(16)	(0. 164)	0. 067–0. 583
(Regional spread)	(7)	(0. 128)	0. 091–0. 198
(Distant spread)	(4)	(0. 149)	0. 066–0. 181
TOTAL	87	0. 149 avg.	0. 060–0. 583
Noncancerous			
Minimal or no disease	52	0. 126	0. 048–0. 213
Postoperative or inflammation	58	0. 139	0. 068–0. 251
Cardiovascular disease	68	0. 161	0. 102–0. 462
Pregnancy	40	0. 153	0. 083–0. 306
Hyperplasia	16	0. 135	0. 091–0. 226
Benign tumor	10	0. 134	0. 083–0. 180
Miscellaneous disease	65	0. 138	0. 076–0. 434
TOTAL	309	0. 143 avg.	0. 048–0. 462
Normal	56	0. 098	0. 043–0. 143

*Expressed in optical-density decrease in 30 minutes.

TABLE 2.—*Distribution of patients in various group subdivisions, according to range of results, expressed in optical-density values*

Group	No. of patients	Values					
		Under 0. 100	0. 100–0. 125	0. 125–0. 150	0. 150–0. 175	0. 175–0. 200	Over 0. 200
Normal	56	29	23	4	0	0	0
Cancerous							
Gastrointestinal tract	23	3	7	5	5	1	2
Leukemia	6	0	0	2	1	2	1
Cervix uteri	11	4	2	0	1	1	3
Lung	4	1	0	0	2	0	1
Prostate	8	1	1	4	2	0	0
Breast	5	1	1	2	0	1	0
Skin	4	1	1	0	1	0	1
Lymphoma	3	1	1	0	1	0	0
Primary site unknown	4	0	0	1	2	0	1
Miscellaneous	19	2	7	6	0	2	2
Noncancerous							
Minimal or no disease	52	7	20	18	2	4	1
Benign tumor	10	2	1	4	2	1	0
Hyperplasia	16	2	6	5	1	1	1
Cardiovascular disease	68	0	12	29	12	3	12
Inflammation	58	7	17	13	12	5	4
Pregnancy	40	2	7	12	11	6	2
Unclassified	65	15	19	16	6	5	4

they mentioned finding normal SLD values in a limited selection of patients with ". . . infections, degenerative, neoplastic and other disease states." They observed elevated SLD in patients with myocardial infarction, diabetic acidosis, acute stem-cell leukemia, chronic myelogenous leukemia, and hepatitis. The present study included 2 patients with acute myocardial infarction of 1 and 3 days' duration—each with abnormally high SLD (0.462 and 0.322). There were no patients with stem-cell or chronic myelogenous leukemia, hepatitis, or diabetic acidosis. The results of this study tend to confirm the work of Wroblewski and LaDue.

SLD activity in mice increased promptly after tumor transplantation and decreased with tumor regression, according to Hsieh, Suntzeff, and Cowdry (*5*). The increased SLD was readily demonstrable 2 or 3 days after transplantation and it increased as the tumor aged. The SLD changes varied with the kind of tumor implanted and the sex of the mice. This contrasts with the results of our study, in which neither a sex difference in SLD between men and women nor a correlation between the extent of the tumors and SLD was observed. These divergent results may be due to the different material used in the 2 studies.

The fundamental basis of this testing procedure presumably depends upon increased production and liberation into the blood of lactic dehydrogenase by cancerous tissue. Hill (*3*) mentioned high lactic dehydrogenase in the cells and fluid of mouse ascites tumors. Except for this, no evidence that cancerous tissue produces excessive amounts of lactic dehydrogenase has been found by us. Relatively high concentrations of the enzyme have been reported in the normal tissues of canine kidney, skeletal muscle, liver, and heart (*4*). As in the study by Wroblewski and LaDue and during the present work, acute destruction of heart muscle was commonly associated with an elevated SLD. It seems unlikely that measurements of an enzyme present in normal tissue can be very useful in cancer diagnosis and management, since the enzyme levels in the serum are governed by complex factors that are not likely to be affected uniformly by neoplasms. These factors include rate of enzyme formation and destruction in the tissues, rate of liberation of the enzyme from the tissue into the blood stream, and the effect of enzyme inhibitors. The relationship between serum acid phosphatase and prostatic cancer, for example, has been studied frequently, and quantitative measurements of this enzyme in the serum are often helpful in the diagnosis and management of prostate cancer, but usually when the disease is so far advanced that only palliative treatment can be offered the patient. The goal, as yet unattained, is the discovery of a serum factor peculiar to cancer tissue so that cancer-selective procedures, based upon qualitative rather than quantitative analytical methods, can be employed.

Summary

Serum lactic dehydrogenase (SLD) was determined in groups of normal persons and cancerous and noncancerous patients in an effort to confirm the cancer selectivity described in a prior report. Patients with and without cancer on the average had elevated SLD values, and no cancer selection

was found. The highest incidence (17.5%) of elevated SLD (over 0.200) was in the group of patients with cardiovascular disease. The 2 patients with acute myocardial infarction had marked SLD elevation, which tended to confirm the work of Wroblewski and LaDue. Prior reports of cancer selectivity by measurements of serum lactic dehydrogenase could not be confirmed.

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An Evaluation of the Chromate-Serum Precipitation Reaction in the Diagnosis of Cancer [1, 2]

J. H. HILL, *M.D.*, *Department of Pathology and Oncology, University of Kansas Medical School, Kansas City, Kansas*

The difference in stability between heated and unheated serum with respect to sodium chromate was reported by Koets (*1*) to be significantly smaller in the serums of cancer patients. When serums from 230 noncancerous and 126 cancerous patients were tested, approximately 9 percent of the noncancerous group had values in the cancer range and about 7 percent of the cancerous group were within the noncancerous range. This selectivity suggested a possible application of the reaction as a serodiagnostic aid in the detection of cancer and stimulated this investigation.

Koets performed the test at 20° C. and determined the amount of precipitate by measuring the height of the column of sedimented protein precipitate. In our first evaluation of the reaction, turbidimetry was employed to measure the amount of precipitate because it was considered more reliable; the tests were made at room temperature, which approximated 32° C. When it became apparent that selectivity for cancer was not being obtained with the modified technique, an evaluation of the method, exactly as described by Koets, was made. An investigation was also carried out to ascertain which of the plasma-protein constituents was responsible for the precipitation observed in the test.

Methods

A. Turbidimetric modification.—The turbidity measurements were made promptly after the reactants had been placed in 12 × 75 mm. round cuvettes and the cuvettes had been inverted twice. A Beckman Model B spectrophotometer was used and the transmission measurements made at a wavelength of 660 mμ. The blank consisted of 5 ml. of Koets' solution A (0.1 *M* sodium chromate) diluted to 100 ml. with solution B (0.1 *M* propionic acid). The tests were carried out at room temperature (32°). The blood was allowed to clot, the serum removed, and the

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test procedure performed within a few hours. Otherwise the procedure described by Koets was followed.

B. Koets' method.—This author's method was followed exactly as published, except that estimates of the height of the protein precipitate could often be made with confidence only to the nearest 0.3 mm., even using a magnifying ocular micrometer, instead of to the nearest 0.1 mm. This was not considered a serious point of difference. As prescribed by Koets, the serum was not removed from the blood clot until after approximately 6 hours at room temperature.

Clinical Material

Blood samples were obtained from fasted patients in the University of Kansas Medical Center. All tests were performed without prior knowledge of the clinical diagnosis. The diagnosis of cancer was made by tissue examination.

A. With turbidimetry technique.—In the group of 39 cancerous patients were 8 men whose average age was 60 years (range 37–73 years) and 31 women whose average age was 53 years (range 29–76 years). In the group of 60 noncancerous patients were 5 men with an average age of 39 years (range 22–56 years) and 55 women whose average age was 40 years (range 19–76 years). The various sites or types of cancerous growths were as follows: uterus, 13; breast, 3; malignant lymphoma, 4; gastrointestinal tract, 10; liver, 1; malignant melanoma, 2; prostate, 3; lung, 2; and kidney, 1. The types of disease in the noncancerous group were as follows: inflammations, 18; cardiovascular disease, 5; benign tumor or hyperplasia, 5; term pregnancy, 11; minimal or no disease, 12; abortion, 3; hepatitis and cirrhosis, 4; diabetes mellitus and aplastic anemia, 1 each. Seven cases with uncertain diagnosis were not used.

B. With Koets' technique.—In the group of 36 cancerous patients were 15 men whose average age was 67 years (range 31–85 years) and 21 women whose average age was 56 years (range 33–80 years). In the group of 103 noncancerous patients were 81 men with an average age of 56 years (range 18–91 years) and 22 women whose average age was 54 years (range 20–88 years). The various sites or types of cancerous growths were as follows: uterus, 9; breast, 3; malignant lymphoma, 4; gastrointestinal tract, 4; urinary bladder, 2; osteogenic sarcoma, 1; prostate, 8; lung, 3; ovary and esophagus, 1 each. The types of disease in the noncancerous group were as follows: inflammations, 25; benign prostatic hyperplasia, 38; minimal or no disease, 35; and miscellaneous, 5. Twenty-seven other cases with uncertain diagnosis were not used.

Serum-Protein Fractionation Procedures

Separation of some of the serum- or plasma-protein constituents was carried out by ultracentrifugation and by the cold-ethanol method, respectively. These fractions were used in place of serum, or were added to the serum used in the test procedure, in an attempt to determine which proteins were responsible for the precipitation.

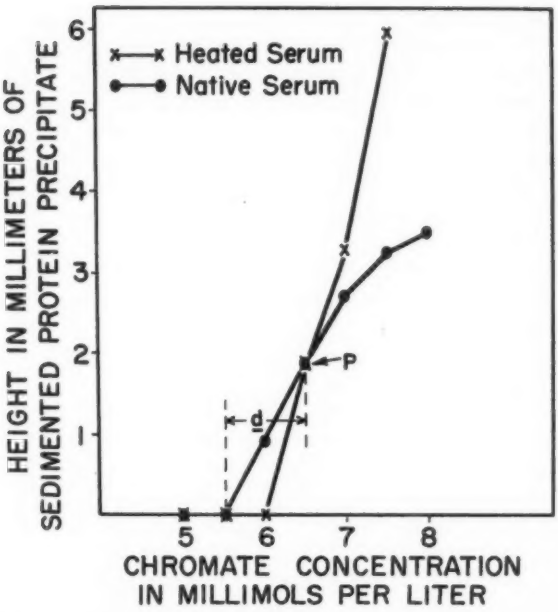
Plasma-protein fractions II, III, IV, and V were separated by an adaptation (*2*) of the cold-ethanol method #10 of Cohn and associates (*3*). The fractions were used in plasma concentration after being dialyzed against 0.9 percent sodium chloride in the cold for 36 hours. Preparation of fraction III was accomplished by removing the protein coagulated by thrombin from fraction I + III. Fraction IV + V was precipitated from fraction IV + V + VI by the modified method (*2*) and fraction V was isolated by method #10 of Cohn and associates (*3*). After dialysis against distilled water at 4 to 8° C. for 48 hours, concentration of fraction V was achieved by lyophilization and was reconstituted to plasma concentration in 0.9 percent sodium chloride. The plasma- and serum-protein fractions, except fraction V, were dialyzed against 0.9 percent sodium chloride because there was evidence that protein precipitation varied with salt concentration. Thus, when 0.1 ml. amounts of increasing concentrations of sodium chloride were added to serum prior to the addition of the chromate reagent, there was progressive decrease in the amount of precipitate, with complete inhibition when 4 percent or stronger salt solution was used.

Separation of a lipoprotein-rich component of serum, the so-called "top fraction," was accomplished by centrifuging 5 parts of serum diluted with 4 parts of 18 percent sodium chloride (specific gravity approximately 1.131) in a Model L Spinco preparative centrifuge at 80,000 × g at 16 to 20° C. for 15 hours (*4*). Besides the lightest oily, yellow, lipoprotein-rich stratum, 3 other strata were recognized grossly, separated, and used in the test in place of whole serum. Beneath the oily surface layer was a water-clear zone blending into a third pale-yellow layer. The fourth and deepest layer was viscid and had an amber color. The second water-clear layer usually contained very little protein, while the third and fourth layers contained the remainder of the serum proteins.

Electrophoretic characterization of the plasma and serum fractions was carried out in an Antweiler Microelectrophoresis Apparatus equipped with a Philpot-Svensson modification of a Schlieren optical-control attachment. Our fraction II was largely γ-globulin, fraction III was mainly lipoprotein-rich α- and β-globulins, fraction IV was chiefly α-globulin, and fraction V almost entirely albumin.

Results

The cancerous and noncancerous groups of patients were compared on the basis of d values, as described by Koets (text-fig. 1). An additional comparison of the 2 groups was made on the basis of the location of point P in terms of mM per liter (text-fig. 1). The cancerous and noncancerous groups tested by Koets' method were also compared on the basis of the location of P in terms of the height of the column of sedimented protein precipitate. A still different basis of comparison of the 2 groups of patients was made when the turbidimetric technique was used and consisted of comparing the distance between the 2 precipitation curves calculated at 5 and 6 mM per liter, expressed in optical-density units

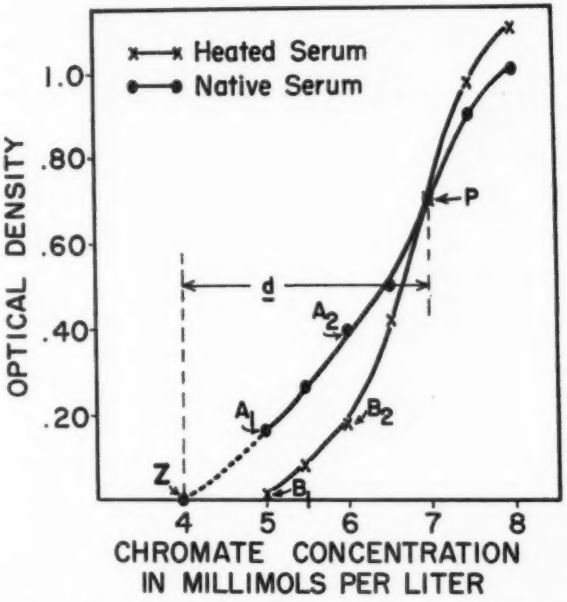


TEXT-FIGURE 1.—Comparison of the amount of sedimented protein precipitate, expressed in mm., produced in heated and unheated serum by sodium chromate of varying concentrations by Koets' method; *d* is difference, expressed in m*M* per liter, between chromate concentrations giving equal precipitation, P, and zero precipitation of the native serum.

(text-fig. 2). These procedures were attempts to establish whether or not the stability difference of the serum proteins with respect to chromate differed in the cancerous and noncancerous groups.

A. Turbidimetry technique.—In 22 of the noncancerous cases and 12 of the cancer cases *d* (text-fig. 2) could not be calculated, because the 2 precipitation curves did not intersect. The mean value of *d* when the curves did intersect was 3.6 m*M* per liter for the 16 serums in the noncancerous group and 3.4 for the 13 serums in the cancerous group. Intersection of the 2 curves (point P) occurred at a mean of 7.0 m*M* per liter in the noncancerous group compared with a mean value of 7.5 in the cancerous group, with much overlapping of results. The mean difference in turbidity obtained with unheated and heated serum from groups of noncancerous and cancerous patients was 0.27 and 0.28 optical-density units, respectively, using the values at 5 and 6 m*M* per liter (text-fig. 2). Cancerous individuals could not be identified by any of the 3 different treatments of the results of this test procedure.

B. Koets' technique.—In 15 of the 103 noncancerous cases and 3 of the 36 cancer cases *d* (text-fig. 1) could not be calculated, because the 2 precipitation curves did not intersect. In the noncancerous group, of 88 cases in which the curves intersected, and in the cancerous group, of 33



TEXT-FIGURE 2.—Comparison of the precipitation, determined by turbidimetry, produced in heated and unheated serum by solutions of sodium chromate of varying concentrations. P is point of equal precipitation; Z is point of zero precipitation of native serum, here obtained by extrapolation; A_1 and B_1 indicate the precipitation produced by 5 m*M* per liter of chromate solution and A_2 and B_2 at 6 m*M* per liter concentration; *d* is the difference between point Z and point P, expressed in m*M* per liter.

cases in which the curves intersected, the mean value of *d* was the same, *i.e.*, 1.1 m*M*. per liter. The mean location of point P in both the cancerous and noncancerous groups was also the same, *i.e.*, 0.68 m*M* per liter, and in the precipitate height range 1.0 to 1.5 mm. Using Koets' criteria of cancer selectivity, *i.e.*, *d* less than 0.6 m*M* per liter, 12 of the 88 noncancerous patients had false positive results and 4 of the 33 cancer patients had true positive results. No correlation between *d* and the types of noncancerous disease or the sites of the cancers was found.

C. Chromate precipitation of plasma-protein fractions.—Indirect evidence was obtained that the precipitation depended principally on the serum lipoproteins. Abundant precipitation occurred when the lipoprotein supernatant obtained by ultracentrifugation was used in the test instead of serum. Relatively little or no precipitate occurred when the other layers were tested. When plasma-strength solutions of plasma-protein fractions obtained by the cold-ethanol method were used instead of serum, abundant precipitation occurred with fraction III (largely lipoprotein), while relatively little, or none, was obtained with fractions II, IV, or V (table 1). Additional evidence of the important role of lipoproteins was obtained by testing serum which had been fortified by addition of the

TABLE 1.—*Results of typical experiment, showing amount of precipitation of sediment obtained with plasma-protein fractions II, III, IV, and V with varying concentrations of chromate reagent*

Plasma-protein fraction	Millimoles per liter chromate		
	7 (*mm.*)	8 (*mm.*)	9 (*mm.*)
II	0	0	Slightly turbid
III	4. 0	40. 0	40. 0
IV	Slightly turbid	0. 5	0. 5
V	0	1. 6	3. 0

TABLE 2.—*Results of typical experiment showing effect of addition of various plasma-protein fractions to serum upon amount of precipitation of sediment with varying concentrations of chromate*

Serum + protein fraction (*0.2 ml. each*)	Millimoles per liter chromate		
	7 (*mm.*)	8 (*mm.*)	9 (*mm.*)
Serum + saline	0. 9	3. 0	3. 7
Serum + fraction II	0. 3	2. 4	3. 0
Serum + fraction III	0. 7	3. 5	8. 7
Serum + fraction IV	0. 3	2. 7	2. 9
Serum + fraction V	0. 3	1. 0	2. 0

various plasma- or serum-protein fractions. For example, the addition of lipoprotein-rich fraction III to serum (table 2) resulted in a definite increase in precipitate, addition of fractions II or IV had little or no effect, and fraction V actually decreased the amount of precipitate. When fraction III was tested alone, a stringy, poorly sedimenting precipitate was obtained. When a mixture of fraction V (albumin) and fraction III was tested, the precipitate was finely divided and tended to settle out as a sediment instead of the stringy, poorly sedimenting precipitate obtained with fraction III alone. Increasing the concentration of albumin led to a decrease or to a complete inhibition of precipitation of fraction III.

Discussion

Koets' results and those of the present study were comparable in the noncancerous but not in the cancerous groups of patients. Thus, in his control group the *d* values ranged between 0.35 and 1.3 m*M* per liter, with a median value of about 0.8, while in this study the noncancerous *d* values ranged from 0.50 to 2.75, with a median value of 1.25 m*M* per liter. These 2 studies differed completely with respect to the *d* values in the cancerous group: Koets found a median *d* value of about 0.35, range 0 to 1.0 m*M* per liter, whereas in this study the cancerous and noncancerous *d* values were almost identical. No explanation could be offered for our failure to obtain differences between the 2 groups. Although the number of patients studied was fewer than reported by Koets, there seemed to be no justification for the accumulation of data on a larger series of patients.

Turbidimetry was used initially to measure the amount of precipitate because most mixtures of serum and low concentrations of chromate did not sediment, and the coarseness of the precipitate obtained with higher chromate concentrations made accurate measurement of the sedimented precipitate impracticable. Since the difference in precipitation of serum proteins by chromate in heated and unheated serum was readily demonstrated by turbidimetry, it seemed reasonable to believe that this method would detect variations, described by Koets, which formed the basis of distinction between cancerous and noncancerous serums. It was realized that sedimentation might indicate a difference in the physical properties of the precipitates that might not be recorded by turbidimetry. Since Koets permitted the mixture of serum and chromate to sediment overnight, the possibility of delayed precipitation was considered; however, turbidimetry readings after an interval of 18 hours revealed little or no change in the *d* readings, although the amount of precipitate was usually increased. At lower temperatures the precipitation was increased and at higher temperatures it was diminished, as described by Koets. This probably accounted for the unsatisfactory results with the sedimentation procedure in the initial work, which was done at a temperature of around 32° C. When the test was carried out at 20° C., as specified by Koets, the sedimentation was sharper. Nevertheless, turbidity without formation of a sediment sometimes occurred.

Although the experiments using serum fortified with plasma-protein fractions, or using the protein fractions in place of serum in the test, indicated that the serum lipoproteins were primarily responsible for the formation of the precipitate, other proteins probably affected the formation of the precipitate also, as illustrated by the inhibitory effect of albumin upon precipitation with fraction III or with serum.

Summary

The stability difference between heated and unheated serum with respect to sodium chromate was not found to be selective when 36 cancerous and 103 noncancerous patients were tested. Evidence was obtained suggesting that the formation of the precipitate in the test depended primarily upon the serum lipoproteins.

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The Effect of Carotene on the Lactic Acid Production and Hexokinase Activity of Hemolysates From Cancerous and Noncancerous Persons [1,2]

J. H. Hill, *M.D.*,[3] *Department of Pathology and Oncology, University of Kansas Medical School, Kansas City, Kansas*

Since glycolysis in cancer tissue leads to excessive lactic acid production compared to normal tissue, efforts have been made to develop a cancer test based on a glycolytic abnormality of blood. Although previous work in this field, summarized in table 1, has not led to a useful means of detecting cancer patients, a reinvestigation seemed warranted of lactic acid production by the blood of cancerous and noncancerous patients and of the effect of carotene on this production. Hemolysates of washed erythrocytes and of washed erythrocytes and leukocytes were used in the first and second experiments, respectively, while in the third experiment, hemolysates of washed erythrocytes were used with added reactants—chiefly adenosinetriphosphate, so that hexokinase activity as well as lactic acid production could be measured and compared in cancerous and noncancerous patients.

I. Effect of Carotene on Lactic Acid Production of Unfortified Hemolysate of Washed Erythrocytes

Preparation of the hemolysate.—Heparinized blood was centrifuged in an International Clinical centrifuge for 15 minutes and the plasma and buffy coat were discarded. The erythrocytes were then washed 3 times in Ringer's solution and centrifuged for 15 minutes between washings, care being taken to remove the buffy layer each time. All these procedures were carried out at room temperature (25° C.). After the third washing, cold distilled water was added to the packed erythrocytes in a ratio of 1.5 volumes of water per volume of erthrocytes. The mixture was then chilled to −1° C., and after 15 minutes a concentrated electrolyte solution was added in 1/12 the volume of the distilled water employed. One hundred ml. of the concentrated electrolyte solution contained 17.8 gm. potassium chloride and 0.5 gm. magnesium chloride. After the addition

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[3] Mr. Samuel W. Greenhouse, Mathematical Statistician, Biometrics Branch, National Institute of Mental Health, gave valuable assistance with the statistical analyses.

TABLE 1.—*Summary of reports dealing with lactic acid (LA) production by blood of cancerous and noncancerous individuals*

Author	Source of blood	Type of blood preparation	Carotene effect	Comment
Ascoli (*1*)	Human patients	Washed erythroctytes		Increased LA production.
Indovina and Fabisch (*2*)	Human patients	Washed erythrocytes		Increased LA production.
Werner and Kleinzeller (*3*)	Rats and mice	Whole blood		Used transplanted tumor; increase, then abrupt fall, and finally elevation in LA production.
D'Allessandro (*4*)	Rats	Washed erythroctytes		Applied tar intraperitoneally; prompt rise in LA production.
Meyer-Heck (*5*)	Human patients	Washed erythrocytes	No stimulation with cancerous blood.	Increased LA production in 76% of cancer cases and 8% of controls.
Wetzler-Ligeti and Willheim (*6*).	Human patients	Whole blood	No stimulation with cancerous blood.	Measured glucose destruction rather than LA production.
Meyer-Heck (*5*)	Human patients	Washed erythrocytes	No significant stimulation or had inhibition using cancerous blood.	Carotene stimulated LA production in 16 of 27 noncancerous patients but in none of 24 cancer patients.
Weis (*7*)	Human patients	Hemolysates	No significant stimulation with cancerous blood.	Significant stimulation of LA production by carotene in cancer-free patients.

of the electrolyte solution, the ingredients were mixed thoroughly and centrifuged for 15 minutes at 6000 r.p.m. in a Sorvall angle-head centrifuge. The clear supernatant constituted the hemolysate.

Test procedure.—The method employed by Weis (*7*), Meyer-Heck (*5*), and Indovina and Fabisch (*2*) consisted of incubating a suspension of erythrocytes, or of hemolysate, with glucose and measuring the amount of lactic acid present in the supernatant fluid. This was compared with the amount recovered from a similarly incubated tube containing carotene, and the difference was considered to represent the effect of carotene. Such a method of estimating the effect of carotene could not be used in this study, since significant lactic acid values were produced by carotene alone and were probably due to some unsuspected ingredient or to traces of alcohol. Consequently, control tubes were always prepared, which differed from the test sample only in that the protein precipitants were added immediately, whereas the test sample was allowed to incubate for the specified time. The amount of lactic acid formed was expressed as the number of micrograms contained in the test flask less the amount recovered from the control flask. The lactic acid determinations were made in duplicate on tungstic acid filtrates by the method of Barker and Summerson (*8*).

To determine the effect of carotene, 0.5 ml. of an aqueous carotene suspension was added to a control and a test flask. The aqueous carotene suspensions were prepared by the method of Aszkenazy, Stern, and Willheim (*9*), using a preparation containing 90 percent β- and 10 percent α-carotene (General Biochemicals, Inc.). This suspension was used immediately to avoid oxidation, which became apparent after 24 hours, particularly if it was not protected from light. The effect of carotene, expressed in μg. of lactic acid, was calculated as follows:

Lactic acid recovered from incubated flask containing carotene *minus* lactic acid recovered from unincubated flask containing carotene

minus

lactic acid recovered from incubated flask without carotene *minus* lactic acid recovered from unincubated flask without carotene.

Clinical material.—Samples of blood from 32 cancerous and 28 noncancerous patients in the University of Kansas Medical Center were tested. The average age of the 12 men in the cancerous group was 65 years (range 19–81) and that of the 20 women was 57 years (range 38–81). The average age of the 20 men in the noncancerous group was 37 years (range 16–69) and that of the 8 women was 49 years (range 20–74). The following types of cancerous disease, all proved by tissue examination, were represented: carcinoma of the cervix, 11; prostate, 6; gastrointestinal tract, 4; breast, 3; bladder, 2; metastatic adenocarcinoma, primary site undetermined, 3; thyroid, 2; and skin, 1. The noncancerous group contained 13 patients with minimal miscellaneous disease, 11 with types of inflammation, 2 normals, and 2 with types of hyperplasia.

Results.—The hemolysates of erythrocytes in the cancerous and noncancerous groups produced approximately equal amounts of lactic acid

and showed similar apparent increase in lactic acid production by carotene (table 2). The increase in mean lactic acid production due to carotene was 44 μg. in the noncancerous group and 51 μg. in the cancerous group. The probability that these effects of carotene were due to chance was less than 0.01 in both the noncancerous and cancerous groups.[4]

TABLE 2.—*Mean values of lactic acid production per flask by hemolysate of erythrocytes from cancerous and noncancerous patients as affected by carotene**

Group	Hemolysate alone (μg./flask)	Hemolysate + carotene (μg./flask)	Differences due to carotene (μg./flask)
Noncancerous.... (28 patients)...	116 (42–239)	160 (78–254)	44
Cancerous..... (32 patients)...	124 (34–286)	175 (93–294)	51

*Figures in parentheses are the range of values.

II. Effect of Carotene on Lactic Acid Production of Unfortified Hemolysate of Washed Erythrocytes *and Leukocytes*

It was decided to include observations on the lactic acid production and carotene effect in hemolysates from which the leukocytes were not removed, since some of the prior workers had used whole blood. The only difference in preparation of the hemolysate was that the buffy layer was not removed each time the erythrocytes were washed. The final hemolysate would thus contain, besides hemolyzed erythrocytes, those products of leukocytes that were liberated by the cold distilled-water treatment. Some leukocytes undoubtedly were not broken down and were removed by final centrifugation. Twenty-four of the 28 noncancerous patients and 20 of the 32 cancerous patients were tested in this manner.

Results.—The cancerous and noncancerous groups were different with respect to the stimulation of lactic acid production by carotene (table 3). The probability was 0.4 that the difference of 9 μg. in lactic acid production due to carotene was by chance in the noncancerous group, but it was only 0.02 that the increase of 56 μg. in the cancerous group was due to chance. The probability was 0.015 that the difference of 47 μg. between the cancerous and noncancerous groups was due to chance.[5]

[4] The following formula for testing the significance of the difference in lactic acid production due to carotene was used:

$$t = \frac{\bar{d} - o}{s_{\bar{d}}}$$

[5] The following formula for testing the significance of the difference of the means of the samples in the cancerous and noncancerous groups was used:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{n_1 + n_2}{n_1 n_2} \cdot \frac{(\Sigma x_1^2 - n_1 \bar{x}_1^2) + (\Sigma x_2^2 - n_2 \bar{x}_2^2)}{n_1 + n_2 - 2}}}$$

TABLE 3.—*Mean values of lactic acid production by hemolysate of erythrocytes* and leukocytes *from cancerous and noncancerous patients as affected by carotene**

Group	Hemolysate alone (*μg./flask*)	Hemolysate + carotene (*μg./flask*)	Differences due to carotene (*μg./flask*)
Noncancerous.... (24 patients)...	164 (86–271)	173 (64–349)	9
Cancerous..... (20 patients)...	144 (75–227)	200 (104–406)	56

*Figures in parentheses are the range of values.

III. Effect of Carotene on Lactic Acid Production and Hexokinase Activity of Fortified Hemolysates of Washed Erythrocytes

Preparation and properties of fortified hemolysate.—Each flask contained 0.2 ml. of 0.5 percent glucose solution, 2.0 ml. of hemolysate of washed erythrocytes, prepared according to directions in section I, 0.1 ml. of a 5.88 percent solution of adenosinetriphosphate sodium (ATP) (Sigma Chemical Company), 0.1 ml. of a 4.08 percent solution of hexosediphosphate magnesium (HDP) (Schwarz Laboratories, Inc.), 0.1 ml. of a 7.33 percent solution of nicotinamide, and 0.1 ml. of a 3.75 percent solution of potassium bicarbonate. The *p*H of this mixture was 7.6. Glucose removal was a rectilinear function of time, and from 50 to 90 percent of the glucose was removed in 60 minutes, depending on the activity of the hemolysate. When glucose was omitted from the system there was a constant rate of production of lactic acid from HDP for 30 minutes, after which no more was formed. When glucose was added to the system, lactic acid formation continued to at least 60 minutes as a rectilinear function of time. Accumulation of glucose-6-phosphate was not a factor limiting glucose removal since it was removed from the test flask at a rate greater than glucose as measured by lactic acid production. This relatively high phosphohexokinase activity in hemolysates corresponded to the results reported by Weil-Malherbe and Bone (*10*).

The hemolysate without ATP showed little or no hexokinase activity. Addition of $KHCO_3$ and HDP enhanced the activity in the presence of ATP. The rate of glucose removal was not increased when more than 2 ml. of the hemolysate was used. Moderate variations in concentration of glucose, nicotinamide, or $MgCl_2$ did not affect the rate. Complete inhibition was produced by addition of 6.3 mg. sodium fluoride to the flask. Slight to moderate inhibition was produced by adding diphosphopyridine nucleotide (DPN) to the flask or by carrying on the reaction under an atmosphere of 95 percent nitrogen and 5 percent carbon dioxide. No change was produced by adding freshly drawn serum, but the activity was greatly increased by addition of leukocytes. No difference in the activity of heparinized and defibrinated blood was found.

In the early experiments the blood was immediately chilled and all procedures were carried out at about 4° C. However, no difference in hexokinase activity was found when the preliminary steps were carried out at room temperature (about 25° C.). Sterile whole blood and hemolysate could be kept at 37° C. for at least 2 hours without significant loss of activity that occurred after 24 hours. Complete loss of hexokinase activity occurred after 5 minutes at 56° C.

Test procedure.—The method of determining hexokinase activity was essentially that used by others to study hexokinase activity in animal tumors (*11*) and rat-brain extracts (*10*). This consisted of glucose estimations, made by the method of Nelson (*12*), on the filtrate obtained after adding to each flask 1.5 ml. of 0.3 *N* barium hydroxide, 9.4 ml. distilled water, and 1.5 ml. of 5 percent zinc sulfate. Besides precipitating the proteins, phosphorylated hexoses were also removed. Hexokinase activity was expressed in terms of the rate of glucose disappearance and was calculated as the percentage of glucose remaining after the incubation period. This was done by dividing the amount of glucose remaining in the test flask after incubation by the amount recovered from the control flask. The control flask was prepared at the same time as the test flask, and the enzyme action was stopped immediately by adding the protein precipitants. The open test flask was permitted to incubate for 60 minutes in a 37° C. water bath before adding the precipitating agents. Duplicate glucose estimations of the filtrate were made, using a 525 mμ filter in a Coleman Model 9 Nephocolorimeter. Lactic acid determinations and addition of carotene were done according to directions in a previous section of this report.

The variations in results of testing the hexokinase activity were found to be relatively small with replicate testing but relatively large with repetitive testing. Thus, the mean coefficient of variation of results was 2 percent (range 0–6.3) when 5 samples of blood were divided into 5 parts and each was tested separately. However, it was 24 percent (range 10–34) when 7 patients were tested daily for 5 days. Investigation of the variations when carotene was added were not made but would undoubtedly be greater than with hexokinase alone, since the result involves calculation of the difference between the results of 2 enzyme test systems instead of 1. Repetitive and replicate testing of lactic acid production was limited to a few observations, which indicated variations in results of the same order as those found in measuring hexokinase activity.

Clinical material.—Hexokinase activity of the hemolysates from 58 cancerous and 63 noncancerous patients in the University of Kansas Medical Center was determined. In 36 of the cancerous group and in 35 of the noncancerous group the effect of carotene on the hexokinase activity was determined. The average age of the 34 men in the cancerous group was 63 years (range 19–86) and that of the 24 women was 52 years (range 33–73). The average age of the 27 men in the noncancerous group was 46 years (range 16–75) and that of the 36 women was 37 years

(range 19–77). The following types of cancerous disease, all proved by tissue examination, were represented: carcinoma of cervix uteri, 11; large intestine, 10; skin and buccal cavity, 7; prostate, 7; lung, 5; breast, 9; malignant lymphoma, 5; larynx, 2; kidney, 1; and brain, 1. The noncancerous group contained 7 normal persons, 5 preoperative patients with minimal disease, 9 postoperative patients with minimal disease, 10 *post-partum* patients, and 2 pregnant patients. There were 17 patients with mild inflammatory processes, 3 with severe inflammation, 2 with cirrhosis of the liver, 3 with arteriosclerosis, 2 with prostatic hyperplasia, and 1 each with thyrotoxicosis, infectious mononucleosis, and thyroid adenoma.

The effect of carotene on lactic acid formation was determined in hemolysates from 32 cancerous and 25 noncancerous patients. In the cancerous group were 10 men whose average age was 63 years (range 39–82) and 22 women with an average age of 53 years (range 30–72). The types of cancer, all proved by tissue examination, were as follows: carcinoma of the cervix uteri, 8; breast, 5; gastrointestinal tract, 5; malignant lymphoma, 4; lung, 2; skin, 2; vagina, maxillary sinus, thyroid, urethra, and ovary, 1 each; and 1 case of multiple myeloma. In the noncancerous group were 9 men whose average age was 48 years (range 16–72) and 16 women with an average age of 45 years (range 21–69). In 11 patients there was evidence of inflammatory processes, 8 had minimal or no disease, and 1 each suffered from arteriosclerosis, toxic goiter, gastric ulcer, cholelithiasis, prostatic hyperplasia, and diabetes mellitus.

Results.—The hexokinase activity of the fortified hemolysates and the effect of carotene in the cancerous and noncancerous groups are outlined in table 4. The difference of the mean values of glucose removed due to carotene was statistically significant, the probability being less than 1 percent that these differences were due to chance. However, when the differences of the mean values of the cancerous and noncancerous groups were tested, the probability was 26 and 34 percent, respectively, that the differences were due to chance. Carotene produced a significant increase in hexokinase activity of hemolysates that was not cancer-selective.

TABLE 4.—*Mean values of hexokinase activity in fortified hemolysates from cancerous and noncancerous patients as affected by carotene**

Group	Fortified hemolysate (*percent*)†	Fortified hemolysate +carotene (*percent*)†
Noncancerous ..... (35 patients)	54 (93–40)	60 (92–46)
Cancerous ..... (36 patients)	56 (94–39)	61 (99–43)

*Figures in parentheses are the range of values.
†Percentage of glucose removed.

Since the effect of carotene has been attributed to its ability to produce anaerobiosis in the glycolytic medium, the following experiments were performed: Four flasks were prepared from each of 22 samples of blood, one without carotene, a second with added carotene, a third containing carotene through which air had been bubbled for 5 minutes, and a fourth without carotene but saturated with 95 percent nitrogen and 5 percent carbon dioxide. Hexokinase activity was measured in the usual manner, with stimulation occurring in most of the carotene flasks and inhibition in 15 of the 22 flasks in which the reaction was carried on under nitrogen and carbon dioxide. Apparently, the carotene effect on hexokinase activity, if due to creation of anaerobiosis in the medium, was different from the anaerobiosis created by carrying on the reaction under nitrogen and carbon dioxide.

Lactic acid production by fortified hemolysate in the cancerous and noncancerous groups and the effect on it of carotene were as shown in table 5. The stimulation of lactic acid production by carotene was not statistically significant since in both the cancerous and noncancerous groups the probability was 20 percent that the differences observed were due to chance. The probability was 55 and 45 percent, respectively, that these differences in lactic acid production between the cancerous and noncancerous groups were due to chance. Stimulation of lactic acid formation by carotene in our preparations was not demonstrated in either the cancerous or noncancerous group of patients. Approximately as much lactic acid was formed by the blood of the patients in the group with cancer as in the group without cancer.

TABLE 5.—*Mean values of lactic acid production by fortified hemolysates from cancerous and noncancerous patients as affected by carotene**

Group	Fortified hemolysate (*μg./flask*)	Fortified hemolysate+ carotene (*μg./flask*)
Noncancerous ..... (25 patients)	338 (211–535)	355 (118–564)
Cancerous ..... (32 patients)	362 (204–621)	389 (194–610)

*Figures in parentheses are the range of values.

Discussion

Separation of cancerous from noncancerous patients could not be carried out in this study on the basis of any of these enzymatic properties of blood cells, except by carotene stimulation of lactic acid formation in unfortified hemolysates of washed erythrocytes and leukocytes. Although statistically significant, this difference between the cancerous and noncancerous groups (*see* table 3) did not appear to be of diagnostic value in view of the considerable overlap of results. The difference was due, possibly, to some

variation in number or kind of leukocytes in the blood of the 2 groups of patients. Leukocyte counts were not done at the time the blood was drawn, but they were performed on 34 patients within a week or less after the time the tests were made. No correlation between the number of leukocytes thus determined and the carotene stimulation of lactic acid production was found in either the cancerous or noncancerous groups. Although the reaction did not appear useful as a testing procedure, the matter will be investigated further.

As outlined in table 1, previous investigators reported no carotene stimulation of lactic acid production with hemolysates from cancerous patients. In this study, hemolysates of erythrocytes from both the cancerous and noncancerous groups showed this change, and when hemolysates of erythrocytes and leukocytes were used, it was the blood from the noncancerous group that did not exhibit carotene stimulation. No explanation for these divergent results can be offered.

The lactic acid blank was considerably higher when carotene was present, possibly due to residual alcohol, which was used in preparing the carotene solution, or to aldehyde groups formed in preparing the carotene suspension. Day-to-day variations in the lactic acid blank occurred but were not a source of error owing to the method used in estimating the enzyme activity. An interesting finding was the moderate decrease in the apparent lactic acid in carotene solutions after 3 hours' incubation at 37° C. This might be due to further oxidation of aldehyde groups in the carotene molecules to carboxyl groups.

The mechanism of carotene stimulation was not studied in detail here, but it has been attributed by others to the creation of anaerobiosis in the medium. However, when the enzymatic reaction was carried on in an atmosphere of 95 percent nitrogen and 5 percent carbon dioxide, slight inhibition of hexokinase activity occurred instead of stimulation.

Lactic acid formation and hexokinase activity of hemolysates did not correlate with the types of cancerous or noncancerous disease in our clinical material. One would expect abnormal glycolytic processes in erythrocytes to be associated with disease of the hematopoietic tissue rather than with disease of other systems of the body. Reid and Ryan (*13*) provided some evidence to support the hypothesis that the presence of a tumor may be associated with an alteration in the erythrocytic enzymes. They found a higher rate of phosphate loss from erythrocytes of cancerous men as compared to normal individuals. In mice with large mammary tumors there was an increased loss of erythrocyte phosphate, which occurred in a space of time too short to be explained by a change in the character of the new cells produced (*14*). Rather, it appeared probable that alteration of the regulating enzyme systems in the erythrocyte had occurred due to some substance elaborated by the tumor. The changes observed by Reid, Ryan, Jones, and Sorenson (*14*) could not be shown to be specific for tumor growth.

Although disease-specific variations in the glycolytic enzymes of erythrocytes have not been found, the serum of cancerous individuals

has been shown to be altered in this respect. Thus, rats bearing the Jensen sarcoma were reported to have elevated serum aldolase and triose isomerase (*15*), while rats bearing sarcoma 39 and Walker carcinosarcoma 256 were found to have elevated serum aldolase (*16*). In human cancer no particular correlation with the clinical findings was noted, with 20 percent of the 104 cases of cancer having elevated aldolase values.

Hexokinase of the rat erythrocyte was studied extensively by Christensen, Plimpton, and Ball (*17*), who found fairly constant hexokinase activity in different rats. The activity of the enzyme from diabetic or hypophysectomized rats was essentially the same as that from normal rats, and addition of insulin or adrenocortical extract had no effect. Inhibition was produced by plasma and did not appear to be due to a nonspecific protein effect. Plasma inhibition was not found with our human hexokinase preparations. Increasing the salt content of their system retarded the reaction, and in the lower total-salt concentrations an excess of K ion with respect to Na was associated with inhibition. The need for fluoride in order to inhibit phosphatase activity was also demonstrated. Although fluoride was not used in our preparations, phosphatase activity did not seem to interfere with the rapid removal of glucose and the constant rate of formation of lactic acid. An important point discussed by these workers is the advisability of employing enzyme systems operating at their maximal rate.

Hexokinase and 2 other enzymes of the glycolytic system were shown by Dische and Ashwell (*18*) to be inhibited by multivalent ions. They worked with hemolysates of pigeon erythrocytes, which are nucleated, and found that inhibition appeared with concentrations of polyvalent ions, which could be considered physiologic. The effect of polyvalent ions in the enzyme system used in this study was not investigated.

Summary and Conclusions

Blood from cancerous and noncancerous persons could not be distinguished on the basis of hexokinase activity, lactic acid production, or carotene effect in fortified and unfortified hemolysates of washed erythrocytes. A statistically significant difference between the cancerous and noncancerous groups was found on the basis of carotene stimulation of lactic acid production by hemolysates of washed erythrocytes and leukocytes, but the overlap of results was such that the difference was not of diagnostic value. The basis of this difference will be investigated.

The carotene stimulation of hexokinase activity of fortified hemolysates was statistically significant, but the effect of carotene on their production of lactic acid was not.

A glycolytic abnormality of blood characteristic of the cancerous state has yet to be demonstrated.

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Urine Fluorescence in Cancer Diagnosis [1,2]

J. H. HILL, *M.D., Department of Pathology and Oncology, University of Kansas Medical School, Kansas City, Kansas*

The present work was suggested by 2 reports that the fluorescence of urine from cancerous patients differed from that of noncancerous patients. In the urine of cancer patients Rabinowitz (*1*) found increased red fluorescence with a low ratio of blue to red (B:R) fluorescence. In benign growth and pregnancy the B:R ratio might be low but the red intensity was not increased. In normal persons the B:R ratio was elevated and the red intensity was not increased. Simon (*2*), using a visual-type fluorimeter, compared cancerous and noncancerous urine on the basis of changes of fluorescence intensity in diluted urine due to ether extraction. He concluded that carcinoma should be suspected if ether extraction of diluted urine resulted in reduced fluorescence, compared to the fluorescence of a similarly diluted but not extracted urine. He found this reaction in 93.8 percent of 81 patients with clinical carcinoma, in 63 percent of 46 patients with latent carcinomas, in 74.1 percent of 27 patients with a family history of cancer, in 63.7 percent of 80 patients suspected of cancer, and in only 4 percent of 470 ill patients in whom cancer was not suspected.

The procedure described by Rabinowitz was duplicated, except for the make of fluorimeter employed. Owing to the low intensity of the fluorescence of diluted urine the procedure of Simon was not followed strictly, *i.e.*, undiluted urine was tested instead of diluted urine and a photoelectric fluorimeter rather than a visual type was used. Neither procedure provided cancer selectivity in this study.

Clinical Material and Method

Clinical material.—The B:R ratio was measured in urine from 10 normal individuals and 35 cancerous and 55 noncancerous patients at the University of Kansas Medical Center. The mean age of the cancerous group (6 men and 29 women) was 53 years (range 21–78), of the noncancerous group (14 men and 41 women), 48 years (range 16–77), and of

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the normal group (5 men and 5 women), 31 years (range 26–36). The following types of cancer were represented: cervix uteri, 10; gastrointestinal tract, 5; metastatic carcinoma, primary site undetermined, 3; breast, 3; corpus uteri, 3; malignant lymphoma, 3; lung, 2; salivary gland, 2; and melanoma, tongue, kidney, and spinal cord, 1 each. Of the 35 cancerous patients, 22 were untreated, 5 had been treated but still had tumor present, 6 were treated, and had no demonstrable tumor, and 2 were in the early stages of radiotherapy. The noncancerous group contained the following types of disease: minimal disease, 6; diseases associated with inflammatory reaction and pregnancy, 30; cardiovascular disease, 6; hyperplasia, 11; and hepatic cirrhosis, 2. The ether-extraction procedure was applied to urine from 24 of the cancerous and 39 of the noncancerous patients, as well as that of the 10 normals.

Method.—The exciting light of the fluorimeter, constructed in this department, was the 365 mμ region of the light of a General Electric AH-4 mercury lamp, isolated by means of a Corning glass filter #5860 and a 10 percent copper sulfate solution with a 4.5 cm. light path. The urine sample was contained in a Beckman quartz fluorescence cell of 10 mm. light path, and the fluorescent light emerging from the bottom of the cell passed through a Wratten 2B filter to exclude scattered ultraviolet light. The filtered light then acted on a type E phototube of a Model 512 Photovolt photometer to cause deflection of the galvanometer, which had first been adjusted to zero reading. The intensity of the blue color of the fluorescent light expressed in galvanometer units, herein designated as B, was measured with a Corning #5543 filter, 5.0 mm. thick, between the Wratten 2B filter and the phototube. These 2 filters together had a peak transmission at a wavelength of 435 mμ. The intensity of the red component, R, was measured, with a Corning #2412 filter 3 mm. thick, which, with the Wratten filter, transmitted light above the 600 mμ wavelength. The mercury lamp was powered through a constant-voltage transformer and was cooled by forced-air circulation. When it was necessary to reduce the intensity of the fluorescent light, neutral-density filters were used after the Wratten filter. Reproducibility was obtained by using a piece of yellow fluorescent glass and a piece of blue fluorescent plastic as standard test materials.

The urine samples were collected in brown bottles containing a small amount of glacial acetic acid; they represented the first urine voided after awakening. All samples were tested within a few hours without prior knowledge of the clinical diagnosis. All cancer patients were classified on the basis of tissue examination.

Ether extraction was carried out by mixing 5 ml. samples of urine with an equal volume of ether and shaking for 1 minute. Transmission measurements were made with a Beckman Model B spectrophotometer.

Results

The distribution of the B : R ratio was essentially the same in the 3 groups of patients, as shown in table 1, *i.e.*, the median of the cancerous

group was in the 12 to 16 range, while the noncancerous- and normal-group medians were in the 16 to 20 range. The ether-extraction procedure usually resulted in increased red and blue fluorescence, as shown in table 2. The median of the increase in red fluorescence readings was in the 0 to 0.5 galvanometer-unit range in all 3 groups of patients. The median increase in blue-fluorescence readings was in the 10 to 20 galvanometer-units range in the cancerous group and in the 20 to 30 unit range in the noncancerous and normal groups, a difference not considered significant. No relationship between these values and the extent, type, or site of pre-cancer was found. Nor was an association between these fluorescent properties of urine and a particular type of noncancerous disease found.

TABLE 1.—*Distribution of ratio of blue : red urine fluorescence (B : R) in 3 groups of patients*

B : R	No. of patients		
	Cancerous	Non-cancerous	Normal
0–4	2	1	0
4–8	5	11	0
8–12	7	7	1
12–16	7	7	3
16–20	5	11	2
20–24	3	6	1
24–28	2	2	1
28–32	3	3	2
above 32	1	7	0
Total	35	55	10

Discussion

Apparently, little is known about the fluorescent material in untreated urine. A characteristic change in urine fluorescence of patients deficient in nicotinic acid was described by Najjar (*3*), but it appeared to have no bearing upon this study. Investigation of the blue fluorescence of urine was made by Kreuzwendedich von dem Borne (*4*), who thought it was related to the indoxyl chemical configuration found in some urine constituents. Of interest was their inability to correlate urine fluorescence with the presence or absence of cancer.

In this study, it was observed that concentrated urine samples had relatively low blue-fluorescence readings due, apparently, to their poor transmittance of blue light. This could conceivably account for low B values. No explanation could be suggested for the elevated R values reported by Rabinowitz. His speculation that porphyrins might account for the apparent red fluorescence could not be substantiated when particular effort was made to extract porphyrins from some of the urine samples, using the fluorimetric methods of Schwartz and associates (*5*).

The increase in B and R values by ether treatment of most urine samples could be explained by the resulting increase in transmittance. This increase, measured at a wavelength of 435 mμ, was as shown in table 3,

TABLE 2.—*Change in red and in blue fluorescence, expressed in galvanometer units, of undiluted urine from 3 groups of patients, due to ether extraction*

Change in fluorescence due to ether extraction	No. of patients		
	Cancerous	Noncancerous	Normal
A. Red			
−3– −2	0	3	0
−2– −1	0	1	0
−1– 0	0	2	1
0–0. 5	14	12	6
0. 6–1. 0	5	12	3
1. 1–1. 5	1	4	0
1. 6–2. 0	3	4	0
over 2. 0	1	1	0
Total	24	39	10
B. Blue			
less than 0	1	1	0
0–10	3	6	2
10–20	9	7	3
20–30	6	15	4
30–40	3	5	0
40–50	2	3	0
over 50	0	2	1
Total	24	39	10

and was not cancer-selective. Since the exact procedure used by Simon was not employed, his results have not been refuted. Preliminary experiments using his procedure (with a photoelectric fluorimeter) showed a consistent increase in total fluorescent light after ether extraction. The present method was adopted because it appeared likely to reveal the alterations Simon described.

TABLE 3.—*Change in transmittance (% T) of urine from 3 groups of patients (wavelength 435 mμ), due to ether extraction*

Change in % T of urine due to ether extraction	No. of patients		
	Cancerous	Non-cancerous	Normal
−8–−4	0	2	0
−4–0	1	1	0
0–4	11	12	3
4–8	4	5	3
8–12	5	7	3
12–16	1	7	1
16–20	0	2	0
over 20	2	3	0
Total	24	39	10

Summary

Urine samples from cancerous and noncancerous patients were compared with respect to their ratio of blue to red fluorescence and to the

change in fluorescence due to ether extraction. Neither property was found to be cancer-selective. There was no evidence that the red-fluorescence component was due to porphyrins. Variation in blue fluorescence was due in part to the degree of concentration of the urine. Ether extraction usually resulted in increased fluorescence due, apparently, to increased light transmittance by the urine.

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